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American Journal of

Veterinary RESEARCH

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Annual AVMA Meeting
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Published bimonthly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Second class postage paid at Chicago, Ill., and at additional mailing offices.

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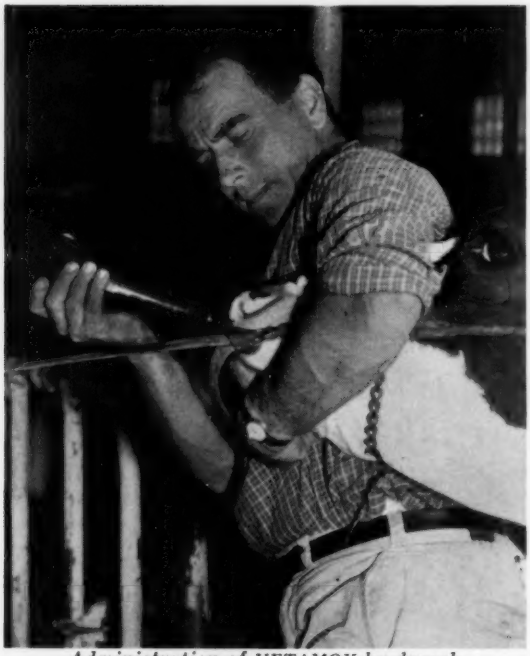

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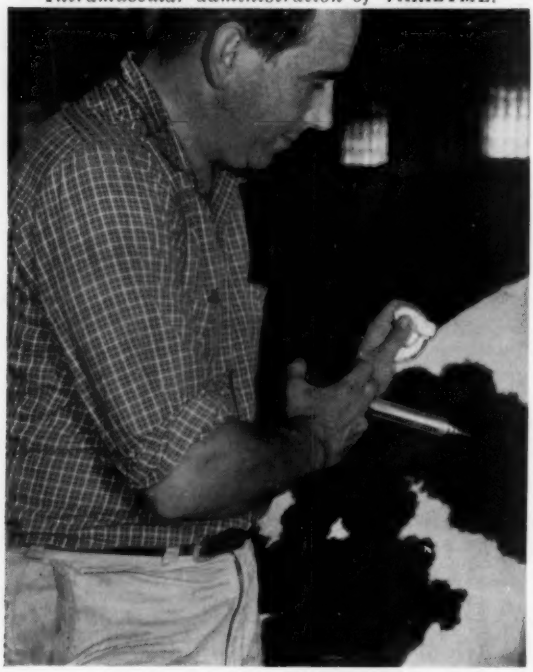

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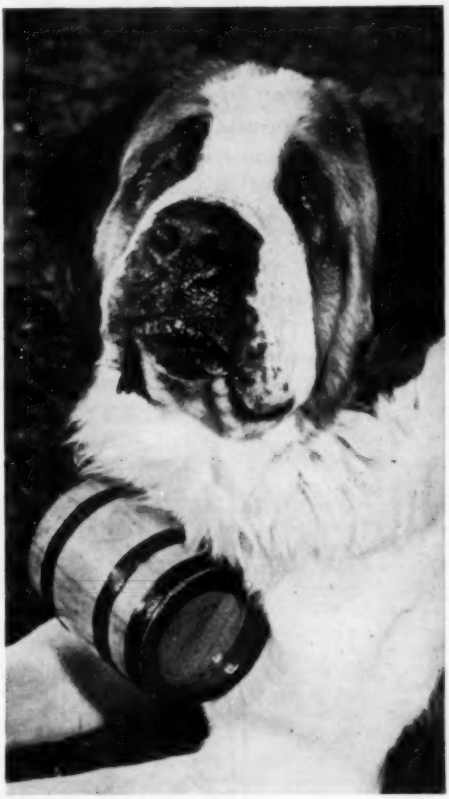

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VOL. XXII JULY, 1961 No. 89

The Relationship of a Feline Virus Isolated by Bolin to Feline Panleukopenia, Kidney Cell Degenerating Virus, and 2 Feline Respiratory Viruses

Daniel Cohen, D.V.M., M.P.H.; David S. Yohn, Ph.D., M.P.H.; Rose A. Pavia, B.S.; William McD. Hammon, M.D., Dr.P.H.

SUMMARY

A virus, isolated by V. S. Bolin and reported to be panleukopenia virus adapted to tissue culture, was immunologically compared *in vivo* and *in vitro* with panleukopenia virus and found not to be the same. A cross comparison of this virus with other feline agents isolated in tissue culture showed this agent to be antigenically different.

INTRODUCTION

While preparing to adapt panleukopenia virus to tissue culture, it was noticed that in 1957 V. S. Bolin [1] reported the adaptation of panleukopenia virus to feline tissue culture. Our attempts to confirm this work and identify his agent are reported here.

Review of Literature

Panleukopenia was named and its pathologic and epizootiologic characteristics were reported by Hammon and Enders in 1939.[5, 6, 9] It was found to be the same clinical entity as the agranulocytosis described independently by Lawrence and Syverton [11] and by Lawrence *et al.*[12] The syndrome described was that of a febrile gastroenteritis and leukopenia occurring 4 to 8 days after exposure. It was associated with a high fatality rate. Intranuclear, acidophilic inclusion bodies were observed in the epithelial cells of the digestive tract as well as in the lymph nodes, bone marrow, and bronchial epithelium. Profound leukopenia was the most characteristic sign of the disease, and the total white blood cell count at times approached a total of a few hundred per cubic millimeter of blood [6, 8, 9, 14, 16] as opposed to a normal range of mean counts of 14,000 to 28,000/cmm.[8]

Hammon and Enders [8] found the rabbit, guinea pig, ferret, and adult mouse insusceptible to infection with panleukopenia virus. Kikuth, Gonnert, and Schweikert are cited by Syverton *et al.*[17] as having found the dog, rhesus monkey, hamster, canary, hedgehog, and rat insusceptible. Syverton *et al.*[17] tried 3 to 6 blind passages and found the rabbit, guinea pig, mouse, and ground squirrel refractory to the virus. Attempts to grow the virus on the chorioallantoic membranes of chicken embryos yielded negative results.[6, 17] Fastier [4] failed to grow the virus in kitten kidney tissue culture. Consequently, the susceptible cat has been the only successful means of detecting the presence of viable virus. Since many cats are immune, due either to a probable prior exposure to virus or the possible presence of maternal antibody, and since uninoculated susceptible cats frequently acquire accidental infection, this *in vivo* system is often unreliable.

In 1957, Bolin [1] reported the adaptation of panleukopenia virus to cat kidney tissue culture. At the 3rd serial passage of an original 10% suspension of splenic tissue from a cat infected with

Received for publication Aug. 24, 1960.

From the Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa. This work was done at the Graduate School of Public Health while Dr. Cohen was an officer of the U.S. Public Health Service with the Veterinary Section, Epidemiology Branch, Communicable Disease Center. His present address is: Wistar Institute, Philadelphia, Pa.

Supported by a grant from the National Cancer Institute.

The authors thank Helen McCray, Della M. Whitaker, and Catherine O'Leary for technical assistance and Dr. Edward O. Gilbert, New Jersey State Department of Health, for the preparation of histologic sections.

panleukopenia, cytopathic effects (CPE) were noticed. Virus from the 4th tissue culture passage was passaged at 24-hour intervals through 16 serial passages. He reported establishing the relationship of the isolate to panleukopenia in 2 ways: (1) The 16th passage virus was tested against antiserums prepared in the rabbit to the original virus-laden splenic tissue. The antiserum neutralized 500 to 1,000, 50% tissue culture infective doses ($TCID_{50}$) of the 16th passage tissue culture virus. (2) The 16th passage material was inoculated into cats and produced a disease clinically similar to panleukopenia. Of the 6 cats inoculated, 4 died from the 15th to 18th days and the other 2 survived.

In 1957, Fastier[4] reported the isolation of an orphan virus from the tissues of a cat with panleukopenia. In attempting to adapt panleukopenia to tissue culture, a virus was isolated which proved not to be panleukopenia virus and was not associated with any disease in the feline host. He called this virus the kidney cell degenerating virus (KCD). The KCD virus, like the Bolin virus, produced CPE within 18 hours. It did not infect any host cells other than those of the cat *in vivo* or *in vitro*. It was distinguished from the panleukopenia virus on the basis of neutralization tests.

In view of the importance of adapting panleukopenia virus to cell culture (in order to have a more suitable laboratory system for its study), and in view of past difficulties,[4] it was considered necessary to confirm the identity of the virus which Bolin had isolated. In this paper, the virus will be referred to as feline viral isolate (FVI).

Materials and Methods

Cell Cultures.—Preparation of monolayers of trypsinized cat kidney cell cultures (CKCC) followed a modification of Youngner's[18] techniques. Weaned kittens, 6 to 8 weeks of age, were exsanguinated and the kidneys removed aseptically and placed into sterile Hanks' balanced salt solution (BSS). After the capsule was removed, the cortex was excised, minced, and washed 3 times in Hanks' BSS and a 4th time in prewarmed 0.25% trypsin solution. The minced tissue was then transferred to a Rappaport trypsinizing flask and 200 to 250 ml. of prewarmed 0.25% trypsin added. The contents were stirred and trypsinized for one hour. The suspended cells were poured off, through sterile gauze, centrifuged at 700 r.p.m. for 5 minutes, and added to growth medium (described later) at the rate of 1 ml. of packed cells per liter of medium. Cells and medium were then dispensed into slanted stationary tubes (1 ml./tube) and incubated at 37 C. After 3 days' incubation, the old medium was discarded and fresh growth medium was added. On the 6th day, when the tissue had fully grown out and was ready for use, the medium was changed to a maintenance medium. The growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks' BSS with 4.0% calf serum and 1.0% antibiotic solution. The antibiotic solution contained 200 units of penicillin, 40 μg. of streptomycin and 10 units of polymyxin per milliliter of medium. The maintenance medium consisted of 0.5% lactalbumin hydrolysate in Earle's BSS with 2.0% calf serum, 1.0% antibiotic solution, glutamine in a final concentration of 2 mM, and 0.1 Gm./100 cc. of $NaHCO_3$.

Virus Sources.—The panleukopenia virus consisted of a 20% suspension of the spleen of a cat (Y.C.) which died of panleukopenia Nov. 25, 1948. One of us (W.M.H.) had used the virus repeatedly in previous studies. This material had been stored at −70 C. until used and was diluted with an equal volume of physiologic saline solution just prior to inoculation of kittens.

The FVI virus was obtained from V. S. Bolin in the form of harvested fluids from infected cat kidney tissue cultures. This material was passaged twice in CKCC in our laboratory. The virus was observed to produce a ++ CPE in 18 hours and a ++++ CPE within 24 hours more. Virus pools were prepared by adding 0.2 ml. of a 10^{-2} dilution of virus suspension to each tissue culture tube containing 1.0 ml. of medium. This was incubated for 24 hours at 37 C., at which time there was a +++ CPE. The fluids were harvested, pooled, sealed in ampules, quick-frozen in an alcohol CO_2 bath, and stored at −70 C. Each pool was titrated in tissue culture. The end points ranged from a $TCID_{50}$ of $10^{5.0}$ to $10^{7.0}$/0.1 ml. when calculated by the method of Reed and Muench.[16] The KCD virus was obtained from Charles J. York in the form of harvested fluids from infected CKCC. This material was passaged once in our laboratory. The virus was observed to produce a ++ CPE in 18 hours and a ++++ CPE within 24 more hours. Virus pools were prepared and titrated according to the method outlined for the Bolin virus.

Two other viruses were obtained from S. H. Madin: (1) feline respiratory virus (FRV), and (2) California feline isolate (CFI). These viruses were also received in the form of harvested fluids from infected CKCC and were passaged once in our laboratory. These viruses produced a ++ CPE in 48 hours. Pools of both viruses were prepared according to the method outlined for the FVI virus with the exception that fluids were harvested in 48 to 60 hours for pooling. All virus pools were tested for bacteriologic sterility.

Neutralization Tests.—Neutralization antibody (NA) tests were performed according to the methods used in this laboratory and described previously.[10] Uninactivated serums obtained from heart blood of kittens under ether anesthesia were used except for specimens obtained from York and Madin and used in experiment 2 described later. York also supplied positive neutralizing antiserum for the KCD virus (Pitman-Moore-RD 180) and Madin supplied convalescent serum specimens for FRV (cat 4) and CFI virus (cat 12). All other serums were prepared in our laboratory. An estimated 100 $TCID_{50}$ inoculum of virus (except where otherwise specified) was mixed with an equal volume of serial fourfold dilutions of serum and incubated for one hour at 37 C. All serums in experi-

TABLE 1—Immunologic Comparison of Panleukopenia Virus and a Feline Viral Isolate (FVI) by Challenge Inoculation of Kittens and Serum Dilution-Neutralization Tests in Cat Kidney Tissue Culture

Kittens		Part I			Part II			Part III		
Litter	Indiv.	Inoc. with panleukopenia virus	Result	NA to FVI virus	Inoculated with FVI virus***	Result	NA to FVI virus	Inoc. with panleukopenia virus	Result	NA to FVI virus
		Day 0	Day 1–29	Day 29	Day 29	Day 30–52	Day 52	Day 53	Day 53+	
A	1	2 cc. i.p.†	Panleuk. recovered	<4‡	3 cc. i.p.	Normal	128	3 cc. i.p.	Normal	128§
A	2	N.D.†	Normal	<4	3 cc. i.p.	Normal	128	3 cc. i.p.	Panleuk., killed day 57	32‖
B	1	3 cc. s.c.†	Panleuk. recovered	<4	3 cc. i.p.	Leukocytosis, ascites	N.D.	Died	.....	....
B	2	N.D.†	Normal	<4	3 cc. i.p.	Leukocytosis, ascites	32	Killed	.....	....

* Panleukopenia virus, 10% suspension of infected cat spleen (yellow cat 11/25/48). ** Neutralizing antibody to feline viral isolate, tissue culture fluids from pool N118 (3rd tissue culture passage of material received from Bolin). *** Tissue culture fluids from pool N117 (2nd tissue culture passage of material received from Bolin). † i.p. = intraperitoneal; s.c. = subcutaneous; N.D. = not done. ‡ Reciprocal of serum dilution calculated as 50% end point. § Serum obtained day 67. ‖ Serum obtained day 57.

ment 1 were tested at one time and in the same lot of tissue culture. The same procedure was followed for experiment 2. In experiment 3, KCD virus *vs.* the FVI was tested on one day and FRV and CFI *vs.* FVI on another.

Blood Counts.—Blood specimens for daily white blood cell counts and differential blood smears were taken from the marginal ear vein. Counts were made before inoculation of virus, daily for 10 days following inoculation and at various intervals thereafter.

Experimental Procedures

Experiment 1 (FVI Virus vs. Panleukopenia Antiserum).—Five feline serum specimens were tested for NA to the Bolin virus (N 117) by serum dilution-neutralization tests in CKCC. Four of these specimens (No. C6956 through No. C6959) were obtained from normal cats used for cell culture. The 5th specimen (No. 12265) was the convalescent serum from a cat experimentally infected with panleukopenia virus. This serum had been maintained for 10 years at −70 C.

Experiment 2 (FVI Virus vs. Panleukopenia Virus).—Two litters of kittens 6 weeks old were obtained from a rural and a suburban area outside the Pittsburgh city limits. These will be referred to as litters A and B. After isolation and observation for 4 weeks (the kittens now 10 weeks old), 2 were selected from each litter. These will be referred to as kittens A-1 and A-2 for litter A and B-1 and B-2 for litter B.

Part I.—On day 0, 1 kitten from each litter (kittens A-1 and B-1) was isolated and inoculated with panleukopenia virus (Y.C.). Kitten A-1 was given 2 ml. intraperitoneally (i.p.); kitten B-1, 3 ml. subcutaneously (s.c.). Kittens A-2 and B-2 were kept strictly isolated from their inoculated littermates in another room of the same building and had their own caretaker. One of us (D.C.) was solely responsible for the care and handling of the infected animals. Separate overshoes and clothing were provided for each room. No equipment from the infected room was allowed to pass into the general circulation until it had been autoclaved, cleaned, and re-autoclaved. Waste materials were taken by the respective caretakers directly to the incinerator. Each room had its own supply of canned cat food and powdered milk. Throughout the experiment, these kittens were maintained in separate rooms. Daily blood counts and smears were made on the inoculated animals for 10 days and on day 29. On the latter day, blood samples were taken from the hearts of all 4 kittens for NA test.

Part II.—Kittens A-1 and B-1 were bathed and sprayed with an insecticide* to eliminate ectoparasites. New cages were provided and the entire isolation room was scrubbed with a disinfecting agent.** All 4 kittens, still maintained as 2 groups

* Gulfspray, produced by Gulf Oil Co., Pittsburgh, Pa.
** Phenolor, produced by E. R. Squibb and Sons, New York, N.Y.

in separate rooms, were then inoculated with 3 ml. of FVI virus i.p. A-2 and B-2 were inoculated first. Blood counts were made daily for 10 days and on the 12th and 14th days following inoculation. On day 52, all 4 kittens were subpected to blood sampling by cardiac puncture. Kitten B-1 died before a specimen could be obtained and littermate B-2 was killed after a blood sample had been obtained.

Part III.—A blood count and smear were obtained from the 2 remaining kitten from litter A and they were inoculated with 3 ml. of panleukopenia virus (Y.C.) i.p. Daily blood counts and smears were made for 10 days on kitten A-1 and 5 days on kitten A-2. On day 57, blood for the NA test was obtained from kitten A-2 and the kitten was killed. A blood sample was obtained from kitten A-1 for NA test on day 67; the kitten was held through day 150 before release.

Experiment 3 (FVI Virus vs. CFI, FRV, and KCD Viruses).—A cross comparison of CFI, FRV, and KCD viruses against the FVI virus by serum dilution-neutralization tests in CKCC was performed to test for possible antigenic relationships.

Results

Experiment 1.—The FVI virus (N 117) was not neutralized at a 1:4 dilution by the normal serums of 4 cats (No. C6956 through No. C6959) or by the convalescent serum of a cat (No. 12265) artificially infected with panleukopenia.

Experiment 2.—The results of the experiment on the relationship of panleukopenia virus to the FVI virus together with details of inoculation and time intervals are summarized (Table 1). In Part I, both kittens inoculated with panleukopenia virus became depressed, lethargic, anorectic, and developed pronounced leukopenia (A-1, 4,250/cmm. and B-1, 2,350/cmm.) on the 4th and 6th day, respectively, following inoculation (Fig. 1). Kitten A-1 had diarrhea for 1 day, 4 days after inoculation, but no diarrhea was recorded for kitten B-1. Illness lasted for about 4 days, whereupon both kittens returned to normal. On day 29, the serums of all 4 kittens had no neutralizing antibodies at a 1:4 dilution for FVI virus.

The 4 kittens were then inoculated i.p. with FVI virus (Part II). All kittens became anorectic for 3 to 4 days following inoculation, A-2 and B-2 having mild febrile reactions (103.4 F.) only for days 2 and 3, respectively. None developed leukopenia; however, the 2 kittens from litter B developed leukocytosis in 6 to 7 days, with pronounced increase of neutrophils. Kitten B-1 had difficulty in swallowing food from the 5th to the 10th day. Ascites developed on the 7th and 10th day in B-2 and B-1, respectively. On the 14th day, 290 cc. of ascitic fluid was removed from kitten B-2. It was milky in color, had no pronounced odor, and was sterile when tested on blood agar plates and thioglycolate broth. It was not extractable by ether but coagulated by acetone. There were mononuclear white blood cells in the sediment. On day 52, 23 days following the FVI virus challenge, kitten B-1 died during cardiac puncture and no blood specimen was obtained. The other 3 kittens were found to have neutralizing antibody titers to FVI virus (Table 1). Kitten B-2 was killed after the blood specimen was obtained and postmortem examinations were performed on kittens B-1 and B-2.

In Part III, kitten A-2, which had not been exposed previously to panleukopenia, developed the disease in 5 days following inoculation, whereas its immune littermate A-1 did not become ill and no leukopenia developed (Fig. 1). Kitten A-2 became lethargic, depressed, and anorectic 4 days after inoculation. On the 5th day, pronounced leukopenia (4,000/cmm.) was observed and the kitten was killed after a blood sample was obtained. Neutralizing antibodies to FVI virus, though reduced in titer (Table 1), were still found at the time

TABLE 2—Cross Comparison of Feline Respiratory Virus (FRV), California Feline Isolate (CFI), and Kidney Cell Degenerating (KCD) Virus Against Feline Viral Isolate (FVI) by Serum Dilution Neutralization Tests in Cat Kidney Tissue Culture

Serum	Virus and dosage			
	N118* FVI	N142 (32 $TCID_{50}$) FRV	N141 (50 $TCID_{50}$) CFI	N156 (100 $TCID_{50}$) KCD
Cat 6—FVI, postinoculation	256	<4	<4	<4
Cat 4—FRV, convalescent	<4	8	N.D.†	N.D.
Cat 12—CFI, convalescent	<4	N.D.	128	N.D.
R 180—KCD virus, postinoculation	<4	N.D.	N.D.	8

* 320 $TCID_{50}$ used in test *vs.* FRV and CFI serums and 5,000 $TCID_{50}$ used in test *vs.* KCD serum. ** Reciprocal of serum dilution calculated as 50% end point. † N.D. = Not done.

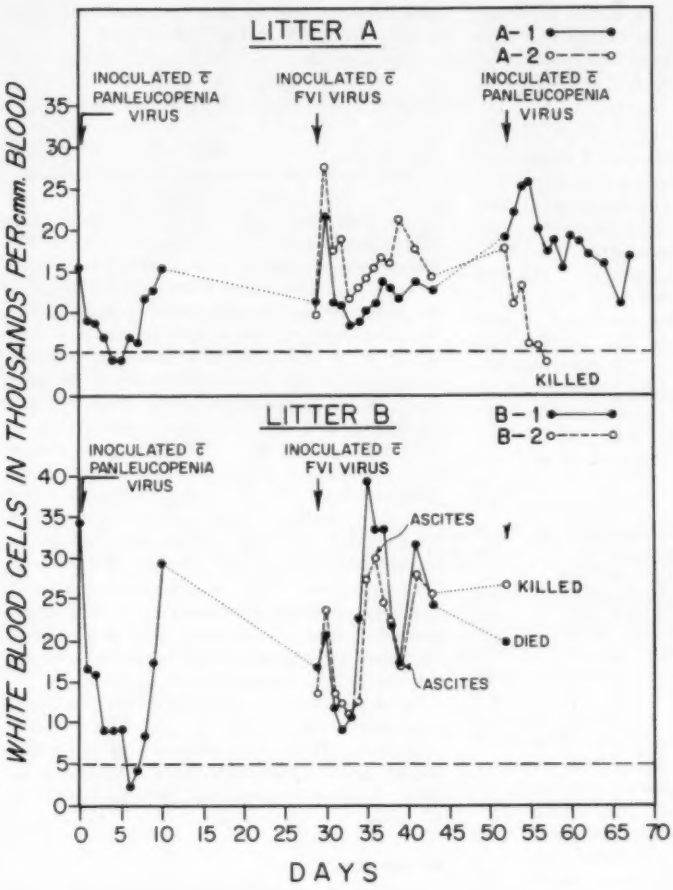

Fig. 1—Total leukocyte counts per cubic millimeter of blood for kittens 1 and 2 of litters A and B, inoculated with panleukopenia and FVI viruses.

the kitten was killed. At necropsy, this kitten was found to have catarrhal enteritis. With the exception of the rectum, the entire gastrointestinal tract was devoid of food and filled with yellowish mucus. Slight bilateral pneumonia of the apical and cardiac lobes was recorded. The bone marrow was beginning to liquefy. On microscopic examination, acidophilic inclusion bodies were found in reticular cells of the spleen, in the mesenteric lymph nodes near the ileocecal junction, in a Peyer's patch, in mononuclear cells of the bone marrow, and in the alveolar cells of the lungs. No inclusion bodies were found in the intestinal epithelium. Some desquamation and necrosis of the intestinal mucosa was observed. The intestinal submucosa was edematous and appeared to be undergoing mucoid degeneration. Its appearance was not unlike that found in the perivascular cuffs of kittens of litter B. The muscularis layers of the intestine were also edematous. These changes were particularly prominent in the lower ileum. The bone marrow contained areas of degeneration and aplasia.

Findings of experiment 2 may be summarized as follows: (1) kittens A-1 and B-1, convalescent from and immune to panleukopenia, failed to produce antibodies capable of neutralizing the FVI virus; (2) presumably susceptible kittens (A-2 and B-2) did not develop panleukopenia when inoculated with the FVI virus; and (3) kitten A-2, after infection with FVI virus, was not protected against a challenge dose of panleukopenia virus.

Experiment 3.—The results of experi-

ment 3 are summarized in Table 2. There was no cross neutralization of the FVI virus with any of the 3 other viruses tested, *i.e.*, FRV, CFI, and KCD virus at a 1:4 dilution.

Discussion

The results of experiment 1 suggested that the FVI and panleukopenia virus are immunologically distinct, for the panleukopenia convalescent serum did not neutralize the FVI virus. Since this serum had been maintained for 10 years at −70 C., the possibility existed that it had lost its NA titer in storage. However, the results of experiment 2 established that the viruses are immunologically distinct. The fact that the FVI virus in Bolin's hands produced panleukopenia in 15 to 18 days after inoculation, as compared with the shorter time intervals observed by Hammon *et al.*,[9] Syverton *et al.*,[17] and Lucas *et al.*,[14] suggests the possibility of a laboratory-acquired coincidental occurrence of panleukopenia in the cats after inoculation with the FVI virus. Such cross infections are difficult to prevent in work with this extremely infectious agent, which possesses remarkable stability.

Bolin apparently isolated a cytopathogenic agent not related to panleukopenia from a cat with that disease. This was a reasonable possibility not without parallel, as illustrated by the experience of Fastier.[4] The circumstances involving the isolation of the Bolin agent suggested a possible relationship with Fastier's KCD virus. Preliminary work by Fastier* showed both viruses had similar gel diffusion patterns. Our NA tests (Table 2) have shown the viruses to be different. The KCD virus was not neutralized by a 1:4 dilution of serum containing neutralizing antibodies to the FVI virus. This test, however, did not rule out the possibility that the viruses are antigenically related in one direction; *i.e.*, KCD-neutralizing antibodies could perhaps neutralize a small amount of FVI virus. The KCD-positive serum used in this test failed to neutralize 5,000 $TCID_{50}$ of FVI virus but had a low neutralizing antibody titer to its homologous virus.

A recent article by Crandell *et al.*[2] showed that FVI virus (referred to as panleukopenia virus) was antigenically different from a number of feline respiratory agents isolated in feline tissue culture. The results with one of these agents, CFI, have been confirmed in our laboratory.

The question of whether the Bolin virus was responsible for the ascites seen in kittens B-1 and B-2 requires further study. On the one hand, all 4 kittens had been inoculated with the same material but only 2 developed pronounced leukocytosis and ascites. The 2 affected kittens had been isolated from each other and continued to be after inoculation with the FVI virus but were from the same litter. However, other members of the same litter not involved in these experiments did not develop ascites. Furthermore, 2 other kittens from a new litter (C), subsequently intraperitoneally inoculated with the FVI virus from the same pool, failed to develop ascites or any type of illness but did develop leukocytosis on the 6th and 8th days. Control kittens were not inoculated with the noninfected cat kidney tissue culture and it is possible that the tissue culture itself may have been responsible for the ascites noticed. All inoculums used were bacteriologically sterile. The FVI virus did not produce a rise in specific antibody titer and, in 6 kittens inoculated, did not produce any evidence of leukopenia, which is the essential characteristic of panleukopenia infection.

It is our conclusion, therefore, that panleukopenia virus was not adapted to tissue culture by Bolin, but that he isolated a new feline agent which appears so far to be distinct from any reported in the literature to date.

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SUMMARIO IN INTERLINGUA

Le Relation Inter un Virus Felin Isolate per Bolin e Virus de Panleucopenia Felin, Virus a Degeneration Reno-Cellular, e Duo Viruses Respiratori Felin

Un virus isolate per V. S. Bolin—reportate como virus de panleucopenia adaptate a histocultura—esseva comparate immunologicamente in vivo e in vitro con virus de panleucopenia e provate como non le mesme. Un comparation cruciate de iste virus e de altere agentes felin isolate in histoculturas provava que illo esseva immunologicamente distincte.

Potency Testing of Low Egg Passage Modified Live-Virus Rabies Vaccines

Donald J. Dean, D.V.M., M.S., and Inez Sherman

SUMMARY

Samples of low egg passage (L.E.P.) modified live-virus rabies vaccines of the Flury type, produced in embryonating chicken eggs and purchased on the open market, varied widely in their ability to infect mice and hamsters and to protect guinea pigs. Since these vaccines met federal specifications when released for sale, the apparent loss of potency presumably reflected either loss of virus activity during shelf life, inadequacies in the testing methods, or both. There is need to determine more accurately the amount of virus required to successfully immunize dogs and to correlate such results with those of tests used to evaluate vaccines. The present guinea pig protection test, while useful, possesses obvious limitations.

INTRODUCTION

Modified live-virus rabies vaccine of the low egg passage (L.E.P.) Flury type, produced in embryonating chicken eggs, has been remarkably successful in controlling canine rabies in New York State.[2] It has been the preferred vaccine in all county-wide vaccination programs since 1952. Until recently, our experience was principally limited to vaccines produced by Company A.[4] As part of our continuing program to evaluate the safety and efficacy of rabies vaccine, however, samples of modified live-virus rabies vaccine produced by 11 additional companies were tested for potency. The results indicated that the virus contents of rabies vaccines vary greatly as measured by their ability to infect mice and hamsters and to protect guinea pigs.

Received for publication Sept. 20, 1960.

From the Division of Laboratories and Research, New York State Department of Health, Albany. The appendix was written by William H. Thompson, Ph.D., at the same location.

Methods and Procedures

Vaccines.—All vaccines were purchased on the open market, stored at 4 C. until used, and tested prior to the expiration date. Each manufacturer was designated by letter.

Protection Tests.—Guinea pig protection tests were made by the methods outlined by the Animal Inspection and Quarantine Division of the United States Department of Agriculture,[11] except that the number of control animals exceeded the suggested minimum and the strength of the virus challenge in 2 experiments was less than recommended. Guinea pigs of the Albany strain (Hartley), weighing 350 to 450 Gm., were used for all protection tests and were observed at least daily throughout the 42-day test period. The protection test consisted of giving 10 or more guinea pigs 0.25 ml. of a 5% suspension of the test vaccine intramuscularly in the thigh. Twenty-one days later, these guinea pigs, together with 5 or more previously selected guinea pigs, were injected intramuscularly in the opposite thigh with 0.5 ml. of the conventionally required dilution of the strain of fixed rabies virus furnished by the National Institutes of Health. Infective mouse brain suspensions, stored at −56 C. until used, served as the source of virus. Customarily, a test has been considered satisfactory when at least 70% of the vaccinated guinea pigs survived challenge without signs of paralysis, and at least 80% of the control animals died or developed clinical signs of rabies within the 21-day observation period.

Infectivity Tests.—Albino mice of the Albany standard strain, weighing 10 to 12 Gm. and selected by stratified random sampling according to sex and weight, were used to determine mouse infectivity titers. Groups of 6 mice each were given, intracerebrally, 0.03 ml. of a succession of fourfold dilutions of the test vaccine judged to cover an adequate range. At the conclusion of the 21-day test period, the median-effective dilution was taken as titer *(T)* as calculated by the moving-average interpolation method of Thompson.[7, 9] A span *(K)* of 3 was used or, if necessary to obtain an interpolation, $K = 2$ or 1.

Groups of 6 to 15 albino hamsters (85 to 100 Gm.) of the Albany strain were used for the hamster infectivity tests. Each hamster was given 0.25 ml. of vaccine intramuscularly in the thigh. The observation period was 30 days.

Sodium chloride solution (0.85%), containing 5% normal serum, served as the diluent in all experiments. Animals dying during the 3-day period

TABLE 1—Pathogenicity of Low Egg Passage Modified Live-Virus Rabies Vaccine for Mice and Hamsters

Vaccine	Infectivity for mice* Relative vaccine concentration 1/100	1/400	1/1,600	1/6,400	1/25,600	1/102,400	Log titer	95% confidence interval for log T	Infectivity for hamsters* Tissue concentration (%) 33.3%**	20.0%**	2.0
A	6/6	6/6	4/6	0/6	2/6	0/6	3.35	(2.97,3.74)	.....	12/14 (85.7%)	1/6 (16.7%)
J	5/6	1/6	0/6	0/6	0/6	0/6	2.30	(1.97,2.64)	2/15 (13.3%)	.....	.....
C	4/6	2/6	2/6	0/6	0/6	0/6	2.30	(1.25,3.36)	1/12 (8.3%)	.....	.....
L	4/5	1/6	1/6	0/6	1/6	0/6	2.28	(2.04,2.53)	0/13 (0.0%)	.....	.....
E	0/6	0/6	0/6	0/6	0/6	0/6	<2.00	<(..... ,2.53)	0/13 (0.0%)	.....	.....

* Numerator = No. of animals reacting; denominator = No. of animals inoculated.
** Vaccine diluted as recommended for field use.

immediately following vaccination or challenge were excluded in evaluating the tests.

Experimental Procedure and Results

Vaccine samples from 5 different manufacturers were tested initially for ability to infect mice intracerebrally and hamsters intramuscularly (Table 1). In the mouse tests, virus was not demonstrated in vaccine E and, whereas vaccine A had a titer *(T)* of $10^{3.35}$, the other 3 vaccines had titers of $10^{2.30}$, $10^{2.30}$, and $10^{2.28}$, respectively. Estimates of 95% central confidence intervals for log *T* are given.[7] In the hamster tests, all vaccines were reconstituted as for field use or further diluted tenfold. Vaccine A killed 85.7% (12) of the vaccinated hamsters as compared to 13.3% and 8.3% of the hamsters that were given vaccines J and C. There were no deaths in hamsters given vaccines E and L. The so-called [3] "exact treatment" of the corresponding 2 by 2 tables for these data indicates that evidence of higher infectivity for vaccine A relative to each of the others is highly significant statistically: $P<0.0002$, as evaluated by Thompson's [8] tables of the 4-variable *psi*-function. When vaccine A was further diluted tenfold, it killed only 1 (16.7%) of 6 hamsters. These results indicate that commercially available vaccines vary with regard to their ability to infect hamsters and mice.

The relationship between the virus con-

TABLE 2—The Comparative Efficacy of Low Egg Passage Rabies Vaccines to Infect Mice and Protect Guinea Pigs

Date of test (1959)	Vaccine Company	Vaccine Expiration date	Infectivity for mice* Relative vaccine concentration 1/100	1/400	1/1,600	1/6,400	1/25,600	Log titer	95% confidence interval for log T	Guinea pig protection No. vac.	No. challenged**	Mortality No.	Mortality %
5/19	A	9/27/59	.....	5/6	4/6	2/6	1/5	3.52	(2.91,4.13)	11	10	0	0.0
	H	5/19/59	.....	4/6	3/6	0/6	0/6	3.05	(2.57,3.54)	11	11	5	45.5
	I	5/26/59	.....	1/6	0/6	0/6	0/6	<2.60	<(..... ,3.13)	11	11	6	54.5
	K	6/28/59	.....	2/6	0/6	0/6	0/6	<2.60	<(..... ,3.13)	11	11	4	36.4
	Controls		.....	.....	.....	.....	.....	.....	.....	....	13	5	38.5
6/9	D	3/ 5/60	6/6	5/6	5/6	1/6	0/6	3.44	(3.08,3.81)	11	11	6	54.5
	E	1/21/60	4/6	0/6	0/6	0/6	0/6	2.15	(1.87,2.43)	11	11	9	81.8
	G	9/ 4/59	6/6	6/6	4/6	3/6	0/6	3.61	(3.24,3.97)	11	11	8	72.7
	H	8/ 6/59	4/6	2/6	1/6	0/6	0/6	2.30	(1.60,3.00)	11	11	7	63.6
	J	3/24/60	4/5	3/6	1/6	0/6	0/6	2.59	(2.10,3.07)	11	11	9	81.8
	K	7/ 7/59	3/6	1/6	2/5	0/6	0/6	2.00	(1.21,2.79)	11	11	11	100.0
	Controls		.....	.....	.....	.....	.....	.....	.....	....	10	10	100.0
7/16	A	4/ 1/60	5/6	5/6	1/6	1/6	0/6	2.90	(2.44,3.37)	10	10	1	10.0
	B	4/ 1/60	5/6	4/6	2/6	0/6	0/6	2.84	(2.39,3.30)	10	10	5	50.0
	C	4/ 4/60	4/6	0/6	0/6	0/6	0/6	2.15	(1.87,2.43)	10	10	5	50.0
	E	1/21/60	0/6	0/6	0/6	0/6	0/6	<2.00	<(..... ,2.53)	10	10	7	70.0
	F	10/ 1/59	5/6	3/6	2/6	1/6	1/5	2.75	(2.16,3.34)	10	10	3	30.0
	G	12/15/59	5/6	6/6	0/6	0/6	0/6	2.84	(2.70,2.98)	10	10	3	30.0
	I	10/ 8/59	4/6	4/6	3/6	1/6	0/6	3.00	(2.21,3.79)	10	10	2	20.0
	L	4/13/60	5/6	2/6	0/6	0/6	0/6	2.42	(2.07,2.77)	10	10	5	50.0
	Controls		.....	.....	.....	.....	.....	.....	.....	....	10	7	70.0

* Numerator = No. of animals reacting; denominator = No. of animals inoculated.
** Same virus pool used for challenge, respectively, in 40-, 20-, and 30-fold dilution.

tent and the ability of a vaccine to protect guinea pigs against rabies was then explored. Eighteen additional samples of vaccine from 12 companies were titrated in mice and simultaneously assessed for their ability to protect guinea pigs (Table 2). As in the previous test, virus was not demonstrated in 1 of 2 vaccines obtained from Company E. The titers of the other vaccines varied from about $10^{2.0}$ to $10^{3.6}$. The mortality from the virus challenge in 2 of the 3 guinea pig tests (5/19/59 and 7/16/59) was less than the minimum 80% prescribed by federal regulations, while the challenge dose in the other test (6/9/59) contained 4 LD_{50} as determined by titration of the challenge virus in guinea pigs. Despite the low level of challenge in 2 of the 3 tests, 13 (72.2%) of the 18 vaccines tested failed to protect 70% or more of the guinea pigs. Certain vaccines failed to afford any protection. The 2 vaccines from Company A afforded the best protection in the 2 guinea pig tests in which they were included.

Samples of 5 different lots of vaccine from Company A were next titrated in mice (8/13/59) and, using a different protection test, simultaneously tested for their ability to protect guinea pigs against rabies. Guinea pigs to be vaccinated as in the earlier experiments, together with previously selected nonvaccinated control guinea pigs, were allotted by random sampling into groups containing 3 animals each and challenged with a succession of twofold dilutions from 1:10 to 1:160 of the CVS strain of rabies virus. Following the 21-day observation period, the results were calculated according to the moving-average interpolation method of Thompson (Table 3).

The mouse titers of the 5 vaccines ranged from $10^{3.5}$ to more than $10^{4.4}$. Unfortunately, the virus preparation used to challenge the guinea pigs was not sufficiently virulent to permit estimation of the full protective potency of the vaccines. The results of this test cannot be directly compared with those of the first 3 guinea pig tests, but all 5 vaccines protected against 5.7 times the median-effective dose for the controls. In the combined results for those groups of animals inoculated with the 10-, 20-, and 40-fold dilutions of virus (Table 3), 7 (77.8%) of the 9 nonvaccinated control guinea pigs died, whereas 3 of the vaccines protected all 9 vaccinated guinea pigs and the other 2 each protected all but 1 of 9.

TABLE 3—Efficacy of Vaccines from Company A in Infecting Mice and Protecting Guinea Pigs (8/13/59)

Vaccine expiration date	Infectivity for mice*: Relative vaccine concentration 1/100	1/400	1/1,600	1/6,400	1/25,600	Log titer	95% confidence interval for log T	Guinea pig protection*: Relative concentration of virus challenge 1/10	1/20	1/40	1/80	1/160
9/27/59	6/6	6/6	4/6	2/6	0/6	3.50	(3.15,3.86)	0/3	0/3	0/3	0/3	0/3
4/ 1/60	6/6	6/6	6/6	4/6	4/6	>4.41	>(3.88, ...)	1/3	0/3	0/3	0/3	0/3
4/ 2/60	6/6	6/6	5/6	4/6	0/6	3.81	(3.49,4.12)	0/3	0/3	0/3	0/3	0/3
8/16/60	6/6	5/6	6/6	4/6	2/6	4.11	(3.58,4.64)	1/3	0/3	0/3	0/3	0/3
11/ 2/60	6/6	6/6	6/6	3/6	3/6	4.11	(3.36,4.85)	0/3	0/3	0/3	0/3	0/3
Controls	.....	.....	.....	.....	.....	.....	.....	2/3	3/3	2/3	1/3	0/3

* Numerator = No. of animals reacting; denominator = No. of animals inoculated.

Four of 7 samples of vaccine A tested killed occasional guinea pigs between post-vaccination days 10 and 18. This phenomenon was not observed with the other vaccines.

Discussion

Since all vaccines tested had met minimum federal specifications when released, the apparent loss of potency presumably reflected either loss of virus activity during shelf life, inadequacies in the testing methods, or both. It is essential that a live-virus vaccine be fairly stable throughout its shelf life. That such a high percentage of the vaccines were found to have low potency when tested suggests that virus instability may be a major problem for certain manufacturers.

The median mouse titer of the 28 vials of vaccine tested approximated $10^{2.8}$. The 8 vaccines from Company A had a median titer of $10^{3.7}$ as compared to $10^{2.5}$ for the 20 vaccines from the 11 other companies. Virus was not demonstrated in 2 vials of vaccine from Company E.

Certain vaccines with fairly high mouse titers failed to protect guinea pigs. If qualitative differences do not exist between the virus strains used for vaccine production, the present studies suggest that, although some correlation apparently exists between the mouse titer and the ability to protect guinea pigs, exclusive reliance upon infectivity tests would not be advisable.

Whenever practicable, vaccine safety and potency tests should be made in animals of the species for which the vaccine is to be used. The guinea pig test [5] is useful but not ideal. The test has not been adequately correlated with the ability of a vaccine to protect dogs. Further limitations include the unavailability of inexpensive standardized test animals and the often-experienced difficulty in obtaining a challenge virus capable of regularly producing high mortality when administered by peripheral routes to guinea pigs.

The recommended number of guinea pigs used in the present potency test should be increased, since a vaccine without protective value may pass this test by chance alone 10 times in 100. If, ignoring previous results, such a vaccine is repeatedly tested, the chance is correspondingly increased that at some point it will appear to have passed. Under the present circumstances, it is recommended that 14 vaccinated and 10 control guinea pigs be used in each test. If acceptability of a vaccine requires that at least 10 of the vaccinated animals should survive and at least 8 of the control animals die or develop signs of rabies, then this would reduce the chance that a vaccine without protective value might pass to less than 1 in 55.* The best safeguard against accepting poor material lies not just in the use of such tests, but in establishing a well-controlled process of preparation that is rarely, if ever, found to yield a product that will not be accepted. Such an effect of producer aim has been discussed with regard to tests upon antigen component preparations.[10]

An obvious need exists for a standard reference vaccine, known to protect dogs, which can be used comparatively to evaluate other lots of vaccines. Since the criteria of infecting mice or protecting guinea pigs have been poorly correlated with the ability of a vaccine to protect dogs, there is urgent need to determine both the minimum and the recommended immunizing dose of modified live-virus rabies vaccine for dogs, and to correlate the results of such standardization with those of methods used to evaluate vaccines.

The results of the present studies were made available to the Animal Inspection and Quarantine Division of the United States Department of Agriculture in October, 1959. Vaccine standards and testing procedures were promptly revised.[1, 12] The revised standards should do much to assure satisfactory potency throughout the authorized shelf life of this product.

* In appendix I, Thompson has offered an alternative suggestion of criteria for *acceptance* or *rejection* of a vaccine from such test results.

APPENDIX I

An Alternative Evaluation System for Rabies Vaccine Protection Tests

William R. Thompson, Ph.D.

With the same experimental system as recommended in the text, starting with 14 vaccinated and 10 control animals, an alternative system is suggested that would further reduce the type 1 risk that a vaccine having no protective potency when so tested would be accepted. There would also be a smaller type 2 risk that a good vaccine when so tested would be rejected. In any case, the type 1 risk is not the same as the risk that a vaccine preparation without protective potency will be submitted to test and accepted. The risk would be zero if no such preparation were ever submitted for test, and the best safeguard against this is to establish a well-controlled process of preparation so that the product is rarely, if ever, not found acceptable.

It is convenient to consider the test results as represented in the conventional 2 by 2 form (Table 4), indicating symbols

TABLE 4—Protection Test Results, Represented in the Conventional 2 by 2 Form

Animals	Survivors (non-reactors)	Reactors	Totals	Survival rate*
Vaccinated	a	b	n′	p′
Control	c	d	n	p
Totals	s	r	N	....

* $p' = a/n'$ and $p = c/n$, the fractions surviving; n′ is the number of nonrejected vaccinated animals and n is the number of nonrejected control animals; b and d are the respective numbers of these that react critically as stipulated.

to denote briefly the 4 individual frequencies *(a, b, c, d)* and the usual row totals: n' = number of nonrejected vaccinated animals, n = number of nonrejected control animals, s = total number of nonreactors (survivors), r = total number of reactors, and $N = n' + n = s + r$. Of the n' vaccinated animals, b reacted and a did not; of the n controls, d reacted and c did not. The experienced survival ratios, $p' = a/n'$ and $p = c/n$, are also indicated in the table.

If $p' > p$, the statistical significance of this may be judged by the so-called "exact treatment" [3] of a 2 by 2 table, based upon the probability P that, if there is a collection of s survivors (or white balls) and r reactors (or black balls), a sample of n' individuals drawn at random from the collection would have a or more survivors (or white balls). This probability may be evaluated [6] by $P = \Psi(a-1, b, c, d-1)$, obtainable exactly and to 7-decimal-place approximation from tables [8] of the 4-variable N- and *psi*-functions, if neither $a + b$ nor $c + d$ exceeds 21, or if neither $a + c$ nor $b + d$ exceeds 21. In the present case, $14 \geqq n' = a + b$ and $10 \geqq n = c + d$.

Accordingly, several alternative test-evaluation systems may be considered.

System I, as in the text, requires acceptance if $a \geqq 10$ and $d \geqq 8$. For $n' = 14$ and $n = 10$, this yields $P = 0.01804$, approximately, for $a = 10$ and $d = 8$, so the type 1 risk is less than 1 in 55.

System II, suggested as a possible improvement in evaluation based upon the same experimental plan, is in accord with directions provided with Table 5. The scheme provides that the type 1 risk for any given test is $P < 0.01$. Each test is classified either as X, discard the particular test result; as A, accept the vaccine; as U, provided that no previous test with the given vaccine has been classed as U, then judgment is suspended; or as R, reject the vaccine. Tests are to be made, if practicable, until either A or R results. If R were used instead of even the first U, then the type 1 risk would be less than 0.01. Use of U should only slightly increase the type 1 risk while reducing the type 2 risk considerably. A careful examination of system II indicates that the over-all type-1 risk would be less than 0.00992 in any case, even if no restriction were placed upon repeated use of the U classification. It is not possible to evaluate the type 2 risk for either system, but through case by case comparison the type 2 risk is apparently less for system II than for I.

The experimental procedure is not suitable for assay of protective potency. It is only an observation that the vaccinated animals have lower susceptibility than the controls and that the difference is statistically significant. The difference is highly significant statistically for $P \leqq 0.01$, since concern here is with significance of differences only in one direction, and so a one-sided test is applied.

Without additional expense, as in use of the conventional forms of bioassay, but at somewhat greater risk of rejection of a vaccine preparation, the obstacle to acceptance could be made somewhat more formidable by use of a challenge dose h per cent greater for vaccinated animals than for controls. Then the same formal decision system II could be used to show, with corresponding statistical significance, that a vaccine so accepted had raised the threshold of resistance in vaccinated animals by at least h per cent above that of controls under the experimental conditions.

TABLE 5—Critical Numbers (a′) for Use in Classifying* a Given Test with n (Nonrejected) Controls, of Which Just d React Critically, and n′ (Nonrejected) Vaccinated Animals, of Which Just a Do Not React Critically

Controls		n′				Controls		n′				Controls		n′			
n	d	14	13	12	11	n	d	14	13	12	11	n	d	14	13	12	11
10	10	7	7	7	6	9	9	8	8	7	7	8	8	8	8	8	7
	9	10	9	9	8		8	10	10	9	8		7	11	10	9	9
	8	11	11	10	9		7	12	11	10	10		6	12	12	11	10
	7	12	12	11	10		6	13	12	11	11		5	14	13	12	11
	6	13	13	12	11		5	14	13	12	11		4	14	....	....	....
	5	14	13	12	....		..	....	....	....	....		..	....	....	....	....

* Class any test as X if either $n' < 11$ or $n < 8$; and, likewise, any test with $d = n$ or with $a = n'$, if not classed as A. Class any test as A if $a \geqq a'$ as given for the actual values of n, d, and n'. If $d < n$ and $a = a' - 1$, class as U unless a previous test with the same vaccine preparation has been classed as U. In all other cases, class the test as R. Each test is thus classed unequivocally as either X, A, U, or R. Accept the vaccine if a test is classed A; reject the vaccine if a test is classed R; and, otherwise, make another test, if practicable, until either A or R results.

The value of h, now zero, could be made greater in accord with any improvement in production methods.

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SUMMARIO IN INTERLINGUA

Testage del Potentia de Vaccino de Virus Vive Modificate per Pauc Passages in Ovos

Specimens de vaccinos anti rabie, a virus vive modificate per pauc passages in ovos (del typo Flury), producite in embryonante ovos de gallina e obtenite in le commercio libere, variava extensemente in lor capacitate de inficer muses e hamsters e de proteger porcos de India. Viste que iste vaccinos habeva satisfacite le specificationes federal quando illos esseva admittite al mercato, le apparente perditas in lor potentia reflecteva presumitemente (1) un perdita de activitate virusal durante le intervallo de stockage o (2) inadequatias in le methodos de testage o (3) ambe ille factores. Il existe le desiderato de determinar plus accuratemente le quantitate de virus requirite pro immunisar canes a bon successo e de correlationar le resultatos de tal determinationes con illos del tests usate in le evaluation de vaccinos. Le test a protection de porcos de India que es presentemente in uso—ben que utile—possede obvie limitationes.

Detection of Leptospires in Naturally Infected Dogs, Using Fluorescein-Labeled Antibody

F. H. White, Ph.D.; H. E. Stoliker, D.V.M.; M. M. Galton, Sc.M.

SUMMARY

Leptospires were isolated from the urine of 17 (11%) of 156 dogs from which satisfactory specimens were obtained by bladder tap. Sixteen of the isolates were identified as *Leptospira canicola* and 1 as *Leptospira icterohaemorrhagiae*.

Leptospires were detected by fluorescein-labeled antibody in the urine sediment of 14 dogs from which leptospires were recovered. In 3 of these dogs, leptospires were found only in the 2nd specimen, obtained 7 to 10 days later.

Leptospires were recovered by culture and were identified by silver stain and fluorescein-labeled antibody in the kidney tissue of 2 dogs killed 4 and 6 weeks, respectively, after first being found infected.

Of 193 canine serum specimens examined by macroscopic (Galton) antigens for leptospiral agglutinins, 46 (23.8%) were found positive. Leptospires were isolated from 15 (32.6%) of the seropositive dogs. The predominant serum agglutinins were against *L. canicola*; however, 3 culturally negative dogs carried agglutinins only against *L. icterohaemorrhagiae*, and 1 against *Leptospira ballum* only.

INTRODUCTION

There have been numerous serologic surveys to determine the prevalence of leptospiral agglutinins in dog serum. Many of these studies have been conducted with antigens of serotypes most frequently isolated from dogs, *Leptospira canicola* and *Leptospira icterohaemorrhagiae*. An exception to this is a study in which 7 leptospiral antigens were used to examine the serum of 357 dogs.[8] However, as pointed out by Alexander *et al.*,[2] there have been relatively few attempts to correlate the presence of agglutinins with recovery of leptospires from dogs. In the past it has been difficult to isolate leptospires from living dogs, due to the problem of readily obtaining uncontaminated specimens. Recently, Menges *et al.*[6,7] reported the successful isolation of leptospires from dogs by direct culture of urine obtained by bladder tap.

A report by White and Ristic [10] described the use of the fluorescent antibody technique for identification of leptospires in the urine of experimentally infected calves. Leptospires in urine specimens were fixed and preserved and could be detected for several weeks after collection.

The present report describes the detection of leptospires in the urine and kidneys of naturally infected dogs by means of the fluorescent antibody technique, compared to culture of urine obtained by bladder tap and to the detection of antibodies in the serum of these animals.

Materials and Methods

Preparation of Labeled Antibody.—Hyperimmune antiserum was prepared by immunization of rabbits with *L. canicola* (Ruebush) according to a previously reported method.[10] The agglutinin titer of the antiserum produced was 1:1,600, as determined by the microscopic-agglutination test, using live homologous antigen and the procedure of Alexander.[1] *Gamma* globulin was extracted from the antiserum with cold methanol by the procedure of Dubert *et al.*[3] The microscopic-agglutination titer of the immune globulin was 1:800. The immune globulin was labeled with fluorescein-isothiocyanate according to the method of Riggs *et al.*[9]

Examination of Dogs.—The dogs examined in this study were stray animals from a Florida metropolitan area and were obtained for research

Received for publication Nov. 15, 1960.

From the Department of Veterinary Science, University of Florida, Gainesville. Published as Florida Agricultural Experiment Station Journal Series No. 1206. M. M. Galton is chief, Veterinary Public Health Laboratory Unit, Communicable Disease Center, USPHS, Atlanta, Ga.

This study was supported in part by grant E-2692 from the National Institute of Allergy and Infectious Diseases, USPHS.

The technical assistance of Mary K. Ramsay, Jane H. Fitzgerald, Emmett B. Shotts, Jr., and John P. Snyder is gratefully acknowledged.

and teaching purposes. Upon arrival, the dogs were subjected to an admission procedure, consisting of anesthetization, devocalization, vaccination for rabies, and administration of protective antiserum containing antibodies against infectious hepatitis, canine distemper, and *L. canicola*. Blood specimens were obtained at the time of cephalic venipuncture for administration of the anesthetic, and the serum samples were subsequently examined for leptospiral antibodies. Macroscopic-agglutination tests were conducted with the serums, using commercial* antigens and control antiserums prepared and used according to the method of Galton *et al.*[4] Selected positive serum samples, and all negative serum samples also, were examined for agglutinins by the previously mentioned microscopic-agglutination test, using live homologous antigens.[2]

While the dogs were anesthetized, urine specimens were obtained by aseptic bladder tap through the abdominal wall. One drop of urine was inoculated immediately into a tube containing 10 ml. of Fletcher's semisolid medium, and 3 drops of urine were inoculated into a second tube of medium. The cultures were incubated at 30 C. and were examined weekly by dark-field microscopy for evidence of growth. They were discarded after 30 days of incubation. When leptospires were isolated, the serotype present was identified by cross-agglutination and agglutinin-absorption procedures as described previously.[5]

For fluorescent antibody examination, approximately 15 ml. of urine was added to 3 ml. of physiologic saline solution, buffered at pH 7.7 with trishydroxy methyl amino methane. The buffered saline solution contained enough formalin (40% formaldehyde) to give the added urine specimen a final formalin concentration of 0.6%. The urine specimens were centrifuged, stained, and examined as previously reported for bovine urine by White and Ristic,[10] using labeled *L. canicola* antibody instead of labeled *Leptospira pomona* antibody.

Two of the infected dogs were killed and examined at 4 and 6 weeks, respectively, after being found infected. Kidney tissues and urine were cultured in Fletcher's medium, and tissues also were fixed in 10% formalin for Warthin-Starry silver staining. Impression smears of cross sections of kidneys were made on microscope slides, and after 30 seconds of fixation with absolute ethyl alcohol, the smears were stained in the usual manner with fluorescein-labeled antibody.

Results

Isolation of Leptospires from Urine.—Urine specimens from 166 dogs were cultured. Urine cultures from 10 of the dogs were contaminated, leaving 156 satisfactory cultures. Leptospires were isolated from 17 (11%) of these dogs. Sixteen of the isolates were identified as *L. canicola* and 1 as *L. icterohaemorrhagiae*.

Detection of Leptospires in Urine by Fluorescein-Labeled Antibody.—Urine specimens from 161 of the dogs were examined by the fluorescent antibody technique. Leptospires were observed in the sediment of 11 of the specimens from which leptospires were recovered. In 3 instances where leptospires were recovered by culture but were not observed by fluorescent antibody staining, 2nd specimens, obtained 7 to 10 days after the 1st examination, contained leptospires when examined by the fluorescent antibody technique. In 2 other instances, however, the dogs were not available for a 2nd examination. Leptospires were recovered from 1 urine specimen not examined by fluorescent antibody staining. Leptospires are shown in the urine sediment of a naturally infected dog after staining with fluorescein-labeled *L. canicola* antibody (Fig. 1).

Detection of Leptospires in Kidney Tissues with Fluorescein-Labeled Antibody.—Leptospires were isolated from the urine and kidneys of both animals which were

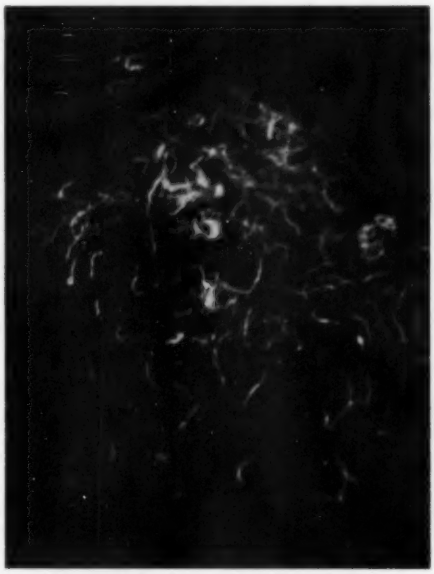

Fig. 1—Leptospires in the urine sediment of a naturally infected dog, as visualized by the fluorescent-antibody method. Photograph taken at approximately x 400, 3-minute exposure on 35-mm. Tri-X film.

* Bacto-Leptospiral Antigens and Antisera, Difco Laboratories, Inc., Detroit, Mich.

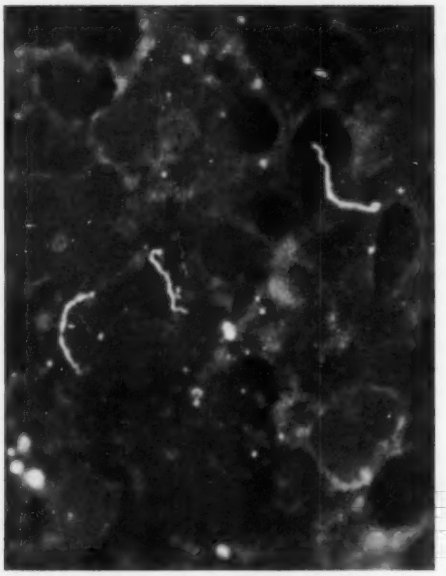

Fig. 2—Leptospires in a kidney impression smear from a naturally infected dog, as visualized by the fluorescent-antibody method. Photograph taken at approximately x 1000, 3-minute exposure on 35-mm. Tri-X film.

killed after being found infected and the organisms were detected in the kidney impression smears by means of fluorescein-labeled antibody. The appearance of leptospires in infected dog kidney tissue as revealed by fluorescein-labeled antibody is shown (Fig. 2). Leptospires were also observed in kidney sections by the Warthin-Starry silver staining technique.

Serology.—Serum specimens from 193 of the dogs were examined for leptospiral antibodies. The serums were first screened with macroscopic-test antigen pools and the results are given (Table 1).

TABLE 1—Agglutination Reactions with Leptospiral Macroscopic-Test Pooled Antigens and 193 Canine Serums

Pool No.	Antigens	No. positive/ No. tested
1	*L. ballum*, *L. canicola*, *L. icterohaemorrhagiae*	44/193
2	*L. bataviae*, *L. grippotyphosa*, *L. pyrogenes*	29/193
3	*L. autumnalis*, *L. pomona*, *L. sejroe*	4/193
4	*L. australis*, *L. hyos*, LT 117: hebdomadis	0/193

Many of the serums agglutinated in both pool 1 and pool 2, mainly due to cross-reactions between *L. canicola* and *L. pyrogenes*.

Serum specimens which were positive in any of the pooled antigens were examined subsequently for agglutinins against the individual antigens in the pools. The number of serums which agglutinated with the individual antigens is shown (Table 2).

The leptospiral agglutinin determinations revealed many cross-reactions between individual dog serum samples and certain of the leptospiral antigens. It was found that when the titer was high for *L. canicola*, positive reactions, usually in a lower dilution, also occurred with one or more of the leptospiral antigens used (Table 2). For

TABLE 2—Agglutination Reactions with Leptospiral Macroscopic-Test Individual Antigens and 193 Canine Serums

Antigen	No. positive/No. tested
L. canicola	39/193
L. pyrogenes	20/193
L. icterohaemorrhagiae	13/193
L. ballum	13/193
L. bataviae	2/193
L. autumnalis	2/193

Of the 89 positive reactions, 14 were against *L. canicola* only, 3 were against *L. icterohaemorrhagiae* only, and 1 was against *L. ballum* only. With 6 serum samples, equal titers were found against *L. canicola* and *L. pyrogenes*. One serum sample had an equal titer for *L. canicola*, and *L. icterohaemorrhagiae*. The remaining positive reactions represent serum samples with highest titers against *L. canicola*, with lower cross-reacting titers against 1 or more of the other antigens.

example, using serologically positive serum from dogs from which *L. canicola* was isolated as positive examples of *L. canicola* infections, it was found that in 12 cases macroscopic-agglutination occurred in dilutions from 1:18.8 to 1:600 (tubes 3 to 9) with *L. canicola* antigen, while reactions with other *Leptospira* antigens *(L. pyrogenes, L. ballum, L. icterohaemorrhagiae,* and *L. autumnalis)* occurred in dilutions from 1:6.5 to 1:325 (tubes 1 to 8), in every case at a lower dilution than with the homologous *L. canicola* antigen. However, serum samples from 3 dogs from which *L. canicola* was isolated agglutinated both *L. canicola* and *L. pyrogenes* antigens at the same dilutions.

In several instances, a doubtful macroscopic-agglutination reaction was observed with undiluted serum specimens from dogs from which *L. canicola* was isolated when they were tested against individual antigens other than *L. canicola*. Subsequent dilutions of these serum samples revealed macroscopic-agglutination titers. This phe-

nomenon may have been due to a prozone reaction.

Serums positive with *L. canicola* antigen in the macroscopic test were positive in titers ranging from 1:100 to 1:6,400 by the microscopic-agglutination test with live antigen. Serums negative to the 12 antigens in the macroscopic-agglutination test also were negative at a dilution of 1:100 by the microscopic-agglutination test when tested against the following serotypes: *L. canicola*, *L. icterohaemorrhagiae*, *L. pomona*, and *L. ballum*.

The serum of the dog from which *L. icterohaemorrhagiae* was isolated was negative by the macroscopic-agglutination test at the time of isolation. However, the serum reacted at a low dilution (1:50) by the microscopic-agglutination test. Seven weeks later, the serum of this dog reacted to *L. canicola* and *L. ballum* antigens by both the macroscopic- and microscopic-agglutination tests, but was nonreactive to *L. icterohaemorrhagiae* antigens. The serum reacted in highest dilution to *L. canicola*, agglutinating at a dilution of 1:32 (tube 4) by the macroscopic test and 1:400 by the microscopic-agglutination test. Leptospires were not isolated by culture or detected by fluorescein-labeled antibody in the urine at this time.

Throughout this study of 193 serum specimens, the predominant serum agglutinins in positive specimens were against *L. canicola*. Exceptions were the serum specimens of 4 dogs from which leptospires were not isolated. Three of these dogs carried agglutinins against *L. icterohaemorrhagiae* only, and 1 against *L. ballum* only.

Of the 193 serum specimens examined for leptospiral agglutinins, 46 (23.8%) were found positive. Leptospires were isolated from 15 (32.6%) of the serologically positive dogs. One isolation was made from the urine of a serologically negative dog, and 1 from a dog whose blood was not obtained for testing.

Discussion

The results of this study indicate that the fluorescein-labeled antibody technique is relatively accurate in the detection of leptospires in the urine of infected dogs. It is postulated that when very small numbers of leptospires are present, the culture of urine obtained by aseptic bladder tap is more reliable. Collection and examination of a 2nd urine specimen from dogs originally negative on fluorescent-antibody examination, but positive on culture, resulted in detection of the organisms. This may have been due to increased numbers of leptospires in the urine at the time the 2nd specimens were taken.

From a clinical standpoint, there are several inherent disadvantages to routine use of the cultural method for the laboratory identification of leptospires. Most important is that a week to a month or more is required for a final isolation. Contamination of urine specimens has been a problem in the past; however, careful use of the bladder-tapping technique would solve this problem. With the fluorescent-antibody technique, a sterile specimen is not necessary and results may be obtained quickly.

From the epizootiologic viewpoint, isolation of leptospires from affected animals is important in order to identify the infecting serotype. Work now in progress is directed toward development of serotype-specific labeled antibodies. If successful, this would permit rapid detection of many specific leptospiral serotypes directly in clinical materials.

The macroscopic antigens, as prepared according to Galton *et al.*,[4] appeared to be generally satisfactory in sensitivity and ease of use. Although a fair number of cross-reactions occurred, they were usually to a much lower titer than the predominating *L. canicola* antibodies. However, there were some fairly high-titered crossings between *L. pyrogenes* and *L. canicola*. The finding that some doubtful reactions were observed with individual antigens against undiluted serums, whereas stronger reactions were obtained in subsequent dilutions of the serums, should be considered in using these antigens. Titrations should be performed when such doubtful reactions are observed.

The diagnosis of leptospirosis in dogs is important, not only from the standpoint of the individual animal, but also in order to block the transmission of leptospires from animal to animal or to man. Leptospires may be shed for long periods by clinically recovered dogs, and thus enhance continuation of the infection in animals. An ideal control program for canine leptospirosis would start with accurate identification of leptospires in the urine of a suspected animal, followed by treatment and isolation of the dog until subsequent urine examinations were negative. This

ideal requires a quick and accurate laboratory diagnostic method. The fluorescent-antibody technique provides a practical means of reaching this goal.

The fluorescent-antibody technique should be conducted in a diagnostic laboratory. The practitioner could collect the urine specimens in laboratory-prepared vials of buffered, formalinized, saline solution for submission to the laboratory.

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SUMMARIO IN INTERLINGUA

Le Uso de Anticorpore Marcate con Fluoresceina in le Detection de Leptospiras in Naturalmente Inficite Canes

Leptospiras esseva isolate ab le urina de 17 (i.e. 11%) de 156 canes ab que satisfacente specimens esseva obtenite per punction vesical. Dece-sex del isolatos esseva identificate como *Leptospira canicola* e 1 como *Leptospira icterohemmorrhagiae*.

Leptospiras esseva detegite per anticorpore marcate con fluoresceina, in le sedimento urinari de 14 canes ab que leptospiras esseva recovrate. In 3 de iste canes, leptospiras esseva trovate solmente in le 2 specimens obtenite 7 a 10 dies plus tarde.

Leptospiras esseva recovrate per culturation e esseva identificate per tincturation a argento e per anticorpore con marcation a fluoresceina in le tissu renal de 2 canes sacrificate 4 e 6 septimanas (respectivemente) post le prime constation de lor infection.

Inter 193 specimens de sero canin examinate per antigenos macroscopic (Galton) pro agglutininas leptospiral, 46 (i.e. 23,8%) esseva positive. Leptospiras esseva isolate ab 15 (i.e. 32,6%) del canes con seropositivitate. Le predominante agglutininas seral esseva agglutininas contra *L. canicola*. Tamen, 3 culturalmente negative canes portava agglutininas contra solmente *L. icterohemorrhagiae* e 1 contra solmente *L. ballum*.

Pulmonary Arterial Lesions in Canine Dirofilariasis

Jerry L. Adcock, D.V.M.

SUMMARY

A histologic study of the lungs of 50 dogs with heartworms revealed marked alterations of the pulmonary arterial tree.

Results indicated that, while embolic dead adult dirofilariae occasionally lead to thrombosis and granulomatous response, the live adults often cause pulmonary endarteritis and obstructive fibrosis. The latter may take the form of specific lesions, described as rugose and villous endarterial fibrosis, which are believed to be of diagnostic significance.

The high incidence of obstructive pulmonary arterial lesions observed supports the view that they play an important role in the development of heart failure in this disease.

INTRODUCTION

This study was undertaken to investigate the morphologic changes in the pulmonary arteries of dogs harboring the canine heartworm, *Dirofilaria immitis*. Effort was also made to determine the relationship of lesions to the presence of either the live or dead adult form of the parasite within affected vessels.

Review of Literature

Otto [4] stated that adult heartworms are usually not found in the pulmonary artery unless their number exceeds the capacity of the right side of the heart. He felt that those that died or were killed by therapy were swept into the pulmonary artery, where they became emboli and caused infarction of the lungs. It was his belief that the signs and lesions of this disease result largely from interference with function of the tricuspid valves due to the presence of the adult parasites in the right atrium and ventricle.

In 1951, Porter [5] described subintimal fibrosis in large branches of the pulmonary artery, endothelial proliferation in smaller branches, and medial hypertrophy in arterioles. He concluded that dilatation and hypertrophy of the right side of the heart are secondary to pulmonary hypertension, but did not relate the arterial changes directly to the presence of adult parasites within the arteries.

Studying arsenical-treated dogs, Balch *et al.*[2] observed granular, pebbly, intimal thickening of the pulmonary artery and its branches. They felt that pulmonary manifestations occur as the adult worms die and become emboli that lead to thrombosis and granulomatous response.

Hennigar and Ferguson [3] described atherosclerosis and endothelial cushions in large branches, endothelial thickening and medial hypertrophy in small branches, and medial hypertrophy in arterioles. They theorized that the endothelial injury caused by the adult parasites might be related to hypersensitivity or mechanical irritation.

Smith and Jones [6] have stated that the cause of the subintimal fibrosis and intimal proliferations is unknown and that their relationship to the parasites has not been clearly established.

Sclerosis and intimal pebbling of the pulmonary artery, calcification and absorption of dead filariae, and thrombosis with infarction have been reported by Bailey [1] in arsenical-treated dogs.

In 1960, Wilcox [7] reported the habitat of adult heartworms to be primarily in the pulmonary arteries, as determined by a series of pulmonary arteriotomies. At necropsy, he observed pulmonary endarteritis and intimal proliferation in 15 of 20 cases.

Materials and Methods

This report is based on materials contributed to the Registry of Veterinary Pathology, Armed Forces Institute of Pathology, Washington, D.C. A total of 50 cases was selected, representing, for the most part, untreated dogs that died or were killed at various stages of the disease. Of the 50 dogs, 6 had been treated with an antimony compound, and 1 had been given arsenical therapy.

All dogs in this series were found at necropsy to harbor adult heartworms. The parasites were usually observed in the pulmonary arteries and in the right atria and ventricles.

Fixed gross specimens of lung were examined in those animals from which such material was still available. Most of the description of gross lesions was, however, obtained from necropsy protocols provided by the contributors.

In addition to routine hematoxylin and eosin, various special stains were utilized.

Customary histologic criteria were utilized in evaluating the viability of the parasites in microscopic sections.

Received for publication Oct. 21, 1960.

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Observations

Gross Pathology.—The most frequent change reported was pulmonary congestion, ranging in pattern from irregular and patchy to diffuse. It occurred in all lobes of some lungs, but was often most severe in the diaphragmatic lobes. The degree of accompanying edema and hemorrhage varied considerably, and infarcts were noticed in a few instances. Fairly well-circumscribed, firm, pale areas, less than 1 cm. in diameter, were distributed throughout the lungs of several animals. Frequently, large numbers of the parasites extended far out into the finer branches of the pulmonary arterial tree. Gelatinous to firm thrombi occasionally were formed about these. In the fixed lungs, careful examination of larger branches of the pulmonary artery frequently revealed dilatation and thickening of their walls. Bronchioles from some animals contained a small amount of cloudy mucinous material.

Dilatation of the right chambers of the heart was reported in only a few cases, even though they were often described as being packed with adult heartworms. It is considered probable that some unrecorded cardiac enlargements may have been present in this series.

Microscopic Pathology.—The more notable types of nonspecific parenchymal lung alterations observed were, in order of frequency of occurrence: congestion, edema and hemorrhage, hemosiderin deposition, interstitial pneumonitis, and infarction.

Macrophages containing hemosiderin were often present in small numbers in the alveoli and interstitial tissue. Heavy and diffuse distribution of these "heart failure cells" was, however, infrequent.

Interstitial pneumonitis was seen as a rather widely distributed, patchy infiltration of the interstitial tissue with a mixture of plasma cells and eosinophils. There was an occasional area of interstitial fibrosis; this change was present to an advanced degree in 2 of the 50 dogs.

Bronchi and bronchioles did not appear to be primarily affected, their contents generally reflecting changes within the alveoli.

By far the most significant lesions observed were those affecting the pulmonary arteries. A classification of these lesions and the number of dogs in which each type was observed is given (Table 1).

TABLE 1—Occurrence of Pulmonary Arterial Lesions in 50 Dogs with Canine Dirofilariasis

Type of lesion	No. of dogs
1) Arteritis	42
a) Inflammatory infiltration of intima ± media	37
b) Proliferation and fibrosis of intima ± media	36
c) Granulomatous response	4
2) Thrombosis	14

Adult parasites, when present in arteries having lesions of type 1a or b (Table 1), were alive. In contrast, those in arteries with the granulomatous response were dead; of 4 dogs in which the worms were dead, 3 had been treated for dirofilariasis.

With the aid of a dissecting microscope, prominent ridges and cushions of intimal

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Fig. 1—Fixed gross lung sample. Pulmonary artery is bisected lengthwise. Upper portion contains heartworms; lower portion has prominent fibrous ridges (arrows), showing that the tissue molded itself about the parasites. x 2.5.

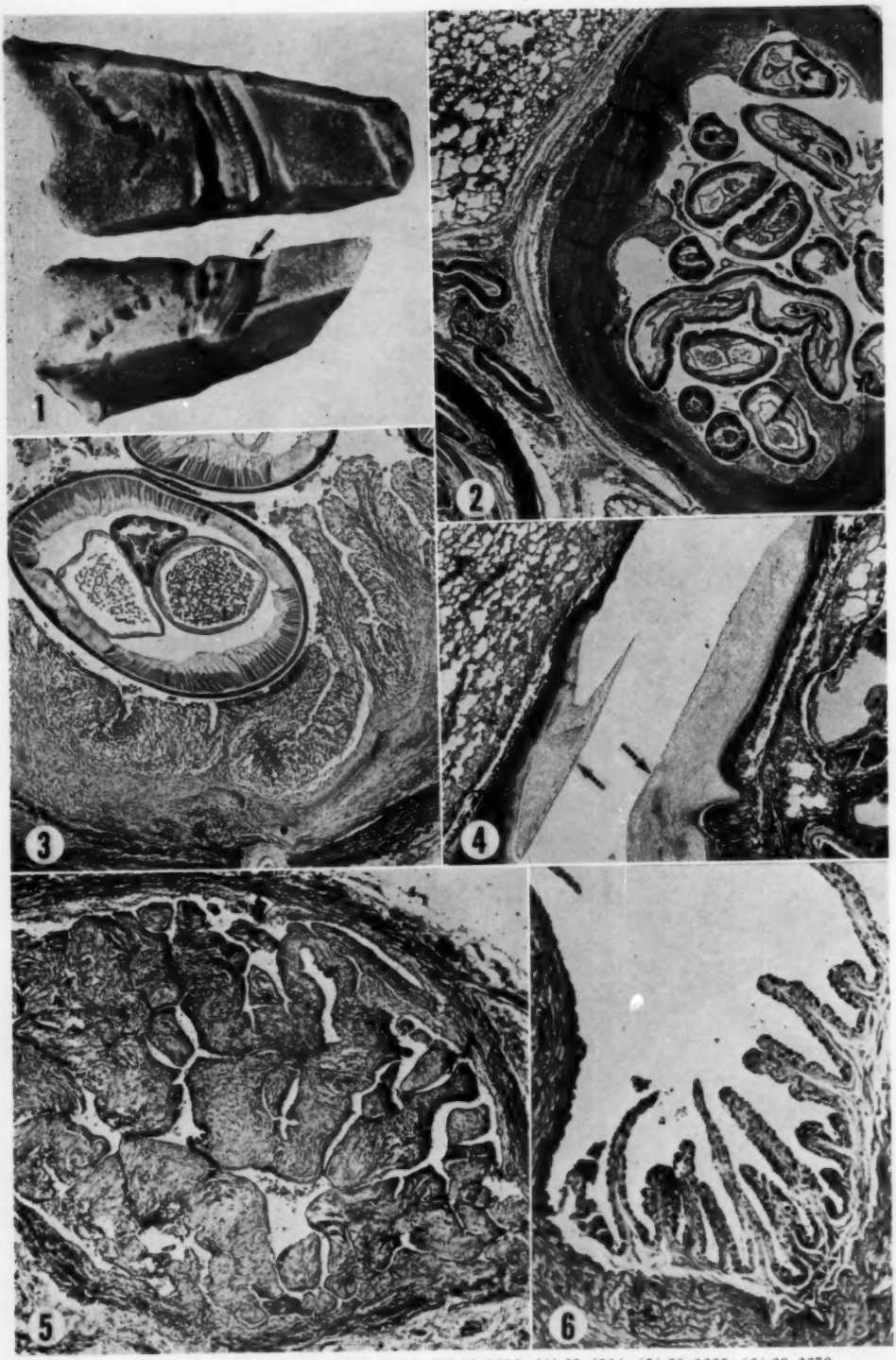
Fig. 2—Pulmonary artery containing live adult filariae. Notice villous appearance of ridges viewed in cross section (arrow). Intimal and medial fibrosis is marked. H & E stain; x 10.

Fig. 3—Higher magnification of portion of artery in Figure 2. Proliferative response around female Dirofilaria immitis, intimal and medial fibrosis, and disruption of inner elastic lamellae (black fibers). Notice crisp morphologic detail of worm which has no degenerative changes. Hart's elastic stain; x 17.

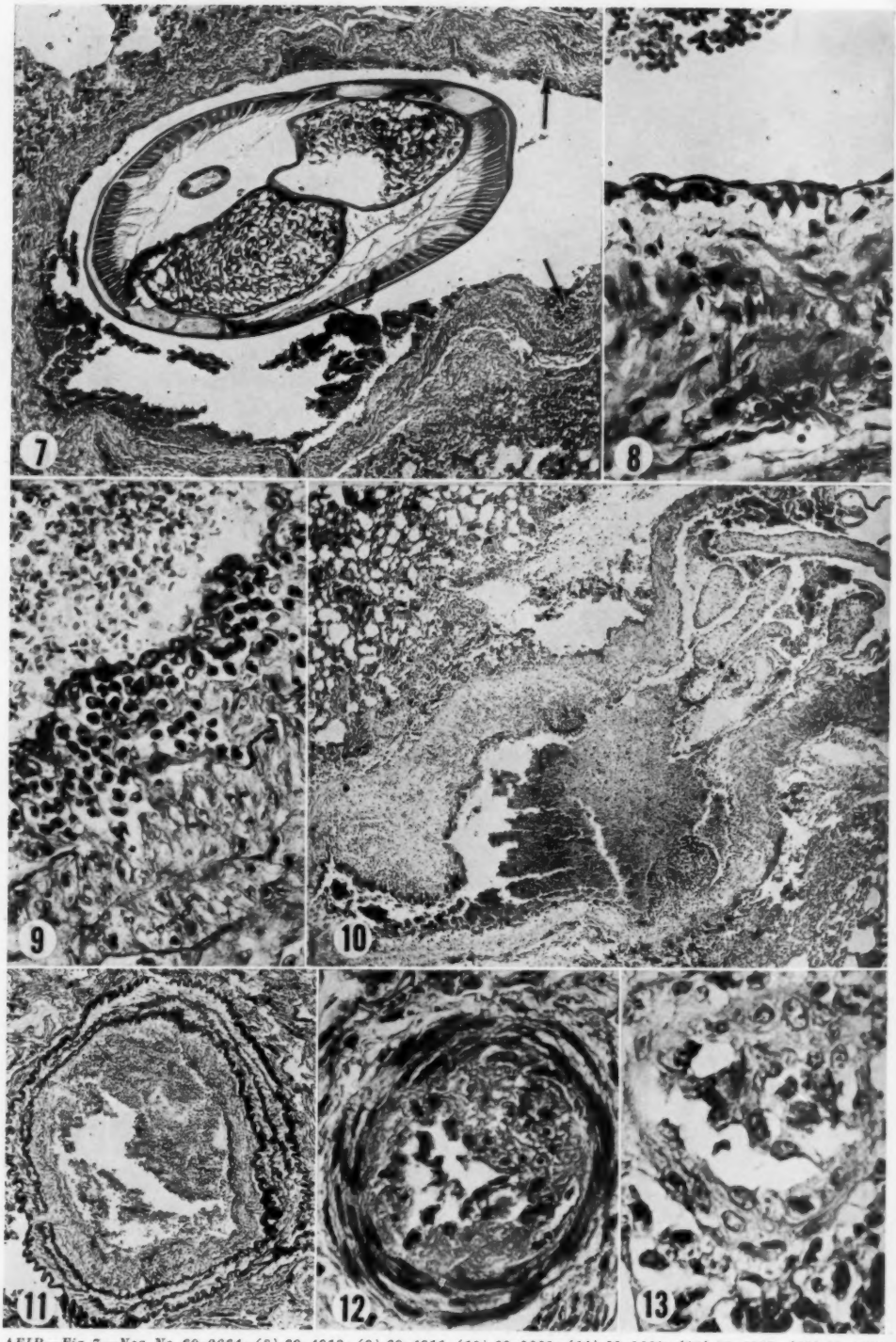
Fig. 4—Fibrous intimal ridges (arrows) sectioned longitudinally. Elastic fibers of media stained black. Hart's elastic stain; x 17.

Fig. 5—Advanced arterial obstruction resulting from maturation of connective tissue composing rugae. H & E stain; x 50.

Fig. 6—Slender villous intimal projections in large elastic artery. H & E stain; x 100.



AFIP—Fig. 1—Neg. No. 59-6962; (2) 60-2674; (3) 60-2676; (4) 60-4014; (5) 60-2666; (6) 60-2670.



AFIP—Fig. 7—Neg. No. 60–2664; (8) 60–4013; (9) 60–4011; (10) 60–2662; (11) 60–2669; (12) 60–4012; (13) 60–2660.

tissue were often seen protruding well into the lumens of thickened arteries. A typical example of this (Fig. 1) shows a specimen of lung divided along the longitudinal axis of the affected artery. In the portion of vessel photographed after removal of the parasites, it is obvious that the proliferating tissue has molded itself about the heartworms, thus accounting for the rugose appearance.

In microscopic sections that included such a large elastic artery in cross section, however, the ridges appeared as villi growing from the inner wall of the vessel into the lumen, as if in an attempt to wall off the offending filariae (Fig. 2). Moreover, such vessels were often greatly increased in their outside diameter and possessed markedly thickened walls.

The ridges themselves were covered by endothelium and composed of plump, loosely arranged fibroblasts and their processes. These cells tended to radiate outward from a central core of dense, relatively avascular connective tissue. This in turn rested upon, and was continuous with, the markedly increased fibrous tissue in the intimal and medial coats (Fig. 3). There was often disruption of the innermost elastic lamellae. The adventitia surrounding the vessel usually was slightly edematous and contained an occasional inflammatory cell. The microscopic appearance of the ridges as seen on longitudinal section is demonstrated (Fig. 4).

A striking feature of these rugose lesions was the fact that they were restricted to the larger elastic branches of the pulmonary artery. These projections varied from broad, luxuriant growths to masses of dense connective tissue resembling the gnarled roots of a tree (Fig. 5). In such advanced lesions there was severe obstruction of the arterial lumen.

In some cases, there was a milder degree of these alterations, with slender, villous intimal projections and little change in the remainder of the vessel wall (Fig. 6). Smaller secondary extensions often proliferated from the larger ones.

While there was some question as to the ultimate fate of these ridges and protuberances, there was evidence of their subsequent destruction by inflammation and necrosis. Subdivision by vascularization and separation of their attachments from the vessel wall also occurred.

The microscopic lesions described in the foregoing paragraphs were seen from the level of the origin of the pulmonary artery down to its smaller elastic branches. At this point, however, a somewhat different response became apparent.

Inflammatory infiltration of the inner aspects of the arterial wall dominated the picture in smaller elastic and large muscular branches (Fig. 7). The earliest changes seen were vacuolation and edema of the subendothelial connective tissue and the presence of small numbers of plasma cells and eosinophils (Fig. 8).

As these changes became advanced, the inflammatory cells increased in number and often penetrated the media. The fibers of the vessel wall became separated and disorganized. Endothelial cells proliferated and produced small convexities projecting into the lumen (Fig. 9). At the same time, inflammatory infiltration of the adventitia became apparent.

At a still later stage, intimal and medial fibrosis participated in this inflammatory

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Fig. 7—Early cellular infiltration (arrows) in an artery containing an adult female heartworm. H & E stain; x 55.

Fig. 8—Beginning endarteritis. Cellular infiltration, vacuolation, and separation of fibers in subendothelial zone. H & E stain; x 350.

Fig. 9—Early endarteritis, more advanced than shown in Figure 8. Endothelial proliferation and infiltration by eosinophils and plasma cells. H & E stain; x 350.

Fig. 10—Advanced cellular infiltration and scarring in a portion of artery in the lower half of the field. Villous-appearing projections in portion at upper right. Notice the fairly abrupt transition between types of lesions. H & E stain; x 45.

Fig. 11—Circumferential intimal fibrosis in a smaller elastic branch of pulmonary artery. Hart's elastic stain; x 80.

Fig. 12—Obstructive intimal proliferation in a muscular branch of pulmonary artery. H & E stain; x 440.

Fig. 13—Pulmonary arteriole, showing intimal proliferation. H & E stain; x 575.

process, producing marked thickening of the arterial wall and large protuberances that encroached upon the lumen (Fig. 10).

Changes of the latter type were often demonstrable in branches leading from large elastic arteries in which the rugose and villous proliferations were present. The transition between the 2 types of reaction (1a and 1b) appeared fairly abrupt. There was no distinct evidence to indicate that inflammatory infiltration preceded the formation of rugose and villous projections in the large arteries. In fact, such infiltration was uniformly slight in the larger elastic branches.

The adult filariae present in arteries affected by both of the foregoing types of lesions were not degenerated. These vessels were all of sufficient diameter to accommodate at least 1 adult heartworm, and the changes appeared to be in response to contact between parasite and arterial intima. Migration or change in position of the heartworms may account for the absence of parasites in some arteries with these lesions.

Inflammation and fibrosis affected the entire inner circumference in branches of such diameter that the entire intimal surface would contact an adult heartworm (Fig. 11).

Small muscular arteries and arterioles often had proliferative intimal and, less often, medial changes (Fig. 12, 13). Occasionally, collections of eosinophils and plasma cells surrounded fragments of microfilariae within arterioles and capillaries.

A granulomatous type of response was present only in those branches of the pulmonary artery that contained dead adult heartworms. These parasites were partially collapsed and fragmented, and there was loss of morphologic detail. The walls of such vessels, as well as the surrounding lung parenchyma, were largely replaced by fibrous connective tissue and chronic inflammatory infiltrate (Fig. 14). Polymorphonuclear leukocytes could be seen invading and destroying some filariae (Fig. 15). In a few instances, the dead parasites had been partially calcified and incorporated into the arterial wall by scar tissue (Fig. 16).

Arterial thrombosis, present in both treated and untreated dogs, occasionally resulted in pulmonary infarction.

An interesting observation with respect to the live adult heartworms is the fact that their digestive tracts contained ingested blood (Fig. 17). This may account, in part, for the anemia reported by clinicians in some cases of dirofilariasis.

Discussion

Analyses of the lesions of canine dirofilariasis have often been limited to the subject of chronic congestive heart failure with dilatation and hypertrophy of the right side of the heart. Some investigators have felt that these changes result solely from mechanical obstruction to blood flow by the space-occupying presence of adult heartworms within the right chambers of the heart and pulmonary arterial tree.

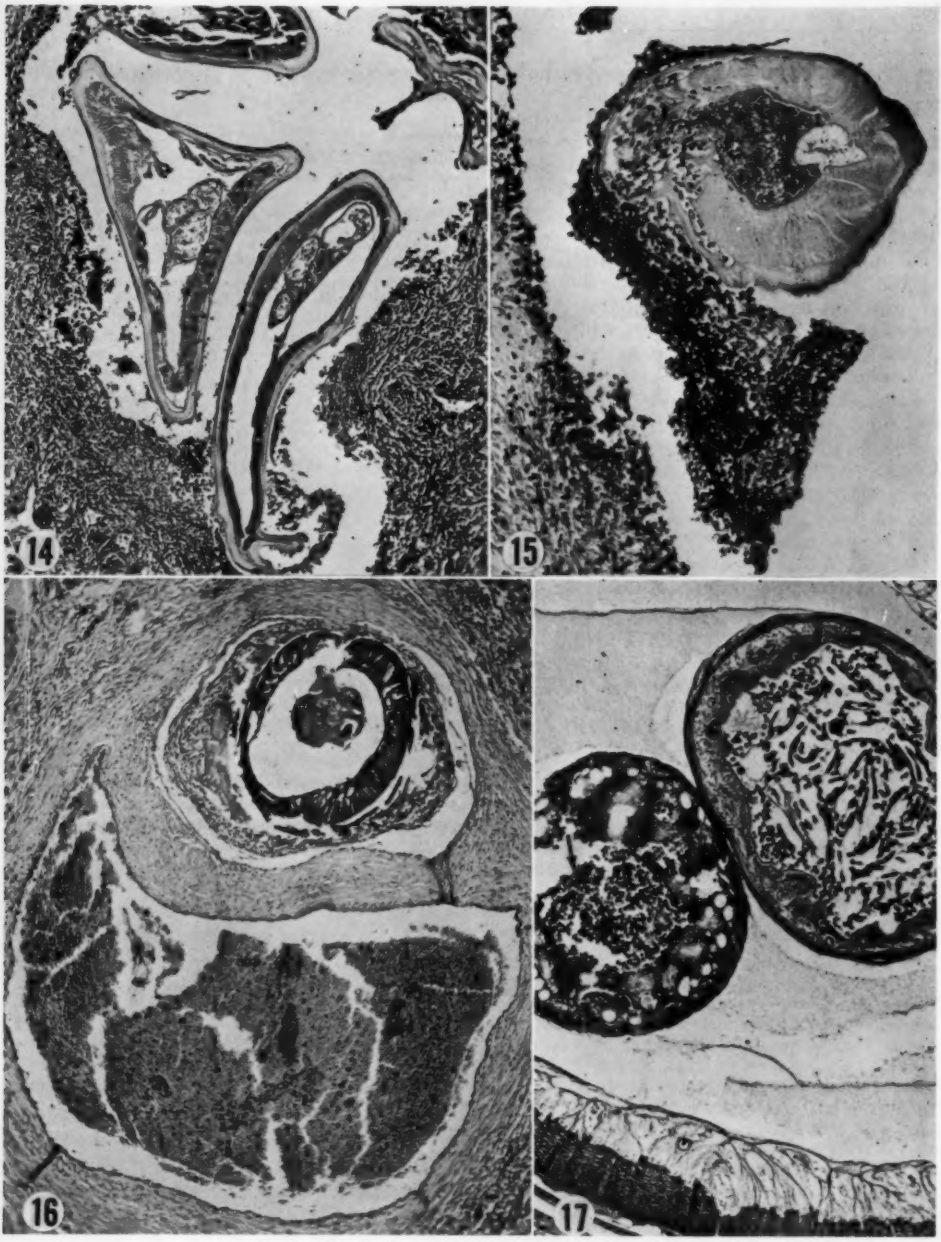
Only in recent years has it been recognized that there are, in addition, severe lesions in the pulmonary arteries of infected dogs and that these changes in themselves may produce pulmonary arterial obstruction, with resultant added stress upon the right side of the heart. For this reason, it seems desirable to emphasize the various types of pulmonary lesions encountered in this disease, particularly those affecting the arteries.

The arterial changes were often considered by previous workers to be the results of embolic dead adult filariae or were dismissed as bearing a suspected, but unconfirmed, relationship to the parasite. Evidence seems ample to support the belief that dead adult dirofilariae may become embolic, leading to thrombosis and granulomatous response. However, knowledge that the live adults may be found in the pulmonary arteries, coupled with histologic evidence resulting from this study, indicates that the live filariae often cause endarteritis and obstructive fibrosis.

In addition to the other arterial changes described in these animals, the finding of unusual lesions that may be described as rugose and villous endarterial fibrosis is considered significant. The author is aware of no other canine disease that produces these types of lesions in the pulmonary arteries.

No explanation is offered for the difference between the response to live heartworms in larger elastic arteries and that observed in smaller elastic and large muscular branches. It is possible that this may be related to the normal structural differences in these vessels.

Changes in small muscular arteries and arterioles are probably secondary to damage in the larger vessels, but may also



AFIP—Fig. 14—Neg. No. 60-2668; (15) 60-2667; (16) 60-2681; (17) 60-4010.

Fig. 14—Granulomatous reaction surrounding degenerating adult parasites in which loss of morphologic detail, collapse, and fragmentation are evident. H & E stain; x 70.

Fig. 15—Invasion of dead heartworm by polymorphonuclear leukocytes. H & E stain; x 130.

Fig. 16—Incorporation of a partially calcified parasite into the wall of an artery (elastic stains established that the space directly surrounding worm is not a separate vessel branching from the large blood-filled lumen at bottom). H & E stain; x 60.

Fig. 17—Ingested erythrocytes (arrow) in digestive tract of heartworm; uterus containing microfilariae seen at upper right. H & E stain; x 140.

result from inflammation stimulated by microfilariae and from thrombosis.

The high incidence of obstructive pulmonary arterial lesions in these dogs supports the belief that this aspect of the disease is highly significant as a cause of eventual failure of the right side of the heart in dirofilariasis.

Clinicians confronted with this disease should consider the probability that they are dealing not only with a parasite that impedes blood circulation, but with severe pulmonary arterial lesions as well, for, even following surgical removal of the heartworms, these lesions remain to exert their influence on pulmonary circulation.

Finally, it is of considerable importance that the true nature and significance of these lesions be recognized in experimental animals.

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SUMMARIO IN INTERLINGUA

Lesiones Pulmono-Arterial in Dirofilariasis Canin

Un studio histologic del pulmones de 50 canes con vermes de corde (= dirofilarias) revelava marcate alterationes del arbore pulmono-arterial.

Le resultatos indica que, durante que morte dirofilarias adulte resulta a vices in thrombosis e un responsa granulomatose, le vive adultes causa frequentemente endarteritis pulmonar e fibrosis obstructive. Iste secunde pote prender le forma de lesiones specific, describite como rugose e villose fibrosis endarterial, e que es reguardate como diagnosticamente significative.

Le alte incidentia de obstructive lesiones pulmono-arterial que esseva observate supporta le these que ille lesiones ha un rolo importante in le disveloppamento de discompensation cardiac in iste morbo.

Homologous Transplantation of Canine Neoplasms

S. W. Nielsen, D.V.M., Ph.D., and C. R. Cole, D.V.M., Ph.D.

SUMMARY

Sixty-seven implants prepared from canine neoplasms were implanted into 48 dogs. Two factors, facilitating the growth of the transplants, were considered in this study: treating the recipients with corticosteroids and with total body irradiation. Two corticosteroids were used: cortisone in a dose of 25 mg. daily to 500-Gm. pups, and prednisolone in doses of 5 and 10 mg. daily or 20 mg. every other day to weanling pups. Thirty-one dogs were irradiated with 170 to 600 r.

In 5 dogs, there was successful growth of the transplant. They were all irradiated, 1 dog with 465, 1 with 500, and 3 with 600 r. In addition, 2 dogs were treated with 10 mg. of prednisolone daily and 1 with 20 mg. of prednisolone every other day. The successfully transplanted tumors were an osteosarcoma, a mixed mammary tumor, and an ovarian adenocarcinoma. The histologic characteristics of the transplanted tumor in the subcutis of the recipients were indistinguishable from those of the donor's tumor.

It can be concluded that depression of the reticuloendothelial system by adrenocortical hormone and total body irradiation were necessary for the successful growth of the canine transplants thus far attempted. Adrenocorticosteroid treatment alone and total body irradiation in doses less than 465 r were insufficient to permit growth of the transplants, but in combination some degree of success was achieved. The most efficient method appeared to be high-dosage radiation at the 465 to 600 r range.

Received for publication Nov. 3, 1960.

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Financial support was received from institutional grants of The American Cancer Society.

INTRODUCTION

Presently, all but 2 of the transplantable tumors available for cancer research are in species such as mice, rats, hamsters, guinea pigs, rabbits, and chickens. Only 2 canine neoplasms have so far been successfully transplanted from dog to dog. The venereal tumor is transplantable by transfer of intact cells into susceptible pups, and it can even be transmitted by natural contact between dogs. The other canine transplantable tumor, an anaplastic carcinoma believed to have originated in the thyroid gland, requires pretreatment of the recipient dogs with total body irradiation and cortisone before successful growth is established after subcutaneous inoculation.

A transplantable tumor in a dog, as opposed to one in a mouse, is a superior tool in research on cancer surgery and therapy because of the larger size of the dog and because of its closer dietary and environmental relationship to man. The objectives of this study were: (1) to attempt homologous transplantation of fresh canine tumors; and (2) to investigate the effects of low-voltage total body irradiation and of corticosteroids on the growth of the transplants.

Review of Literature

The first successful transplantation of a neoplasm from one animal to another has been accredited by Shimkin [20] to a Russian veterinarian, Novinsky. After 44 unsuccessful attempts, the latter succeeded, in 1877, in transplanting 2 canine tumors to other dogs. Both neoplasms had the characteristic morphologic and biologic features of the tumor now known as the canine venereal tumor. One came from the nose of a 4-year-old dog and the other from the vagina of a 6-year-old dog. Seligman,[23] in 1908, stated that the tumor was endemic in New Guinea before the advent of the white man. Since the initial reports, several transmission studies have been made; these were reviewed in 1932 by Feldman.[5] Two additional transmission experiments were performed by Stubbs and Furth [29] in 1934 and by Karlson and Mann [15] in 1952.

The histogenesis of the canine venereal tumor is still disputed. Jackson [14] believes that the tumor is composed of the same cells as are present in the

heart base tumor of the dog. Feldman[5] considers them to be lymphocytic cells. Mulligan[26] states that they are of histiocytic origin, while Bloom *et al.*[3] believe that the tumor cells are mature reticuloendothelial cells. During the past few decades, this venereal neoplasm in its original form has become rarer in this country. Stubbs and Furth[28] noticed this in 1934, when they found only 5 cases in 30,000 dogs examined over a 3-year period at the School of Veterinary Medicine, University of Pennsylvania. They stated that the tumor was much more frequently encountered 10 to 20 years previously. At the College of Veterinary Medicine, Ohio State University, only 2 cases of venereal sarcoma, both of the extragenital form, have been diagnosed in approximately 30,000 dogs examined during the 10-year period from 1949 to 1959.

Various attempts have been made to find other transplantable tumors in the dog. MeWhorter and Prime,[19] in 1916, were able to transplant a mammary chondrosarcoma from an 11-year-old terrier into 5 of 20 untreated recipients. Mann,[18] in 1919, attempted transplantation of a mammary carcinoma and found growth in 3% of the recipients. Only 1 transplant, however, persisted for 41 days; the remainder became necrotic and were resorbed between 7 and 12 days.

Mohiyuddeen and Cole,[21] in 1952, were successful in transplanting 4 different canine tumors to the anterior chamber of the eye in guinea pigs. A total of 41 transplants from various animals were made into 241 guinea pigs. They consisted of 25 malignant, 10 embryonic, and 6 benign tissues. The canine tumors successfully grown were an osteosarcoma, a melanoma, a sebaceous adenocarcinoma, and a seminoma.

Allam *et al.*[1] were unsuccessful in numerous attempts to transplant 13 different canine neoplasms via various routes into pups. After the instigation of total body irradiation and cortisone treatment of the recipients in 1954, they succeeded in the homologous transplantation of an anaplastic tumor removed from the thyroid region. The recipient pups were given from 300 to 500 r in addition to treatment with 2 to 5 mg. of cortisone per pound of body weight from the day of tumor implantation. The transplant has now been carried through 30 generations of dogs.[2]

By "stressing" the recipients with irradiation and cortisone, several human tumors have been transplanted from man into various laboratory animals and maintained during many serial passages.[30] Toolan has been successful in heterologous transplantation of 33 of 100 neoplasms from man to rats and mice which had been given 600 and 450 r, respectively.[29, 31]

Materials and Methods

Seventeen dogs with 12 different neoplasms (Table 1) served as donors. Implantations were performed on 48 dogs, consisting of 10 different litters of Beagles and small terriers. At the time of tumor implantation, their ages ranged from 12 hours to 1 year. The pups were weaned after approximately 6 weeks and subsequently were vaccinated against distemper and hepatitis.

Preparation of the Implants.—Approximately 4 ml. of tumor tissue was removed, placed in a sterile Petri dish, and mixed with 100,000 units of aqueous penicillin, 10 mg. of streptomycin, and 16 ml. of sterile physiologic saline solution. Later in the study, the saline solution was replaced by a balanced nutrient medium designed for tissue cultures.[17, 20] This was done in order to provide the tumor cells with a better environment during the first, critical hours after implantation and before adequate vascularization had developed.

Half of the tumor tissue was minced into fine pieces, measuring less than 1 mm. in their greatest dimension, with sterile scissors and scalpels. The other half was ground in a glass tissue grinder (TenBroeck), which resulted in reduction into clumps of 2 to 50 tumor cells as visualized on smears. The 2 halves were mixed and the fluid

TABLE 1—Summary of Sixty-Seven Homologous Transplantation Attempts with Seventeen Canine Neoplasms

Donor dog	Type of neoplasm	Pretreatment of recipients	No. of implants	No. of "takes"
A761	Mastocytoma	None	6	0
A305	Reticulum cell sarcoma	None	9	0
A1293	Mastocytoma	170 r, 300 r	4	0
A1358	Perianal adenoma	170 r, 300 r	1	0
A1692	Venereal tumor	600 r, prednis.	6	0
A1742	Ovarian adenocarcinoma	600 r, prednis.	3	1
A1779	Thyroid carcinoma	300 r, prednis.	4	0
A1709	Osteosarcoma	300 r, prednis.	4	0
B697	Seminoma	300 r, prednis.	2	0
B723	Chondrosarcoma	300 r, prednis.	2	0
B549	Mixed mammary tumor	465 r,600 r, prednis.	6	3
B424	Seminoma	465 r,600 r, prednis.	6	0
B989	Osteosarcoma	500 r, prednis.	4	1
B1324	Thyroid carcinoma	500 r	1	0
B1355	Granulosa cell carcinoma	500 r	1	0
B1375	Mastocytoma	500 r	1	0
B1480	Lymphoma	400 r, cortisone	7	0

was aspirated with a 20-ml. syringe, leaving all tough fibrous strands in the container. Within 20 minutes after removal from the donor, the tumor cell suspension was injected subcutaneously into the groins of the recipients through a 14-gauge needle.

Preparation of Cell Cultures of Tumors for Implantation.—Tumor tissue was excised aseptically from the donor, minced with a sterile pair of curved scissors and scalpels, and processed according to the tissue culture method described by Griesemer.[11]

Attempts were made to transplant cell cultures from 3 different neoplasms, a mixed mammary tumor (B549), a seminoma (B697), and an ovarian granulosa cell carcinoma (B1355). Three additional tumors, used in direct transplantation attempts, were grown in tissue cultures in order to determine their viability: a seminoma (B424), a chondrosarcoma (B723), and a lymphoma (B1480).

Preparation of the Recipient.—Two treatments of the recipient favoring successful transplantation were considered in this study: (1) adrenal cortical hormones, and (2) total body irradiation.

1) Treatment with adrenal cortical hormones was carried out alone or in conjunction with total body irradiation to depress the inflammatory reaction and the antibody formation of the host against the implanted tumor cells. Cortisone acetate was given in doses of 25 mg. per day to 500-Gm. pups and prednisolone* in doses from 5 mg. to 10 mg. daily to weanling pups. Prednisolone has approximately a 5 times greater antiphlogistic effect than cortisone without having the accompanying undesirable sodium- and water-retaining effect. The treatment was initiated the day before irradiation or 1st tumor inoculation and was continued until death or for a maximum of 3 weeks.

2) Irradiation was used alone, or in combination with corticosteroids, as a method of reticuloendothelial system ablation in an attempt to destroy the antibody-forming cells of the body. Total body irradiation in this study was used on 31 dogs in graded doses of 170, 300, 400, 465, 500, and 600 r for the purpose of determining the optimal dose permitting successful growth of the implants without killing the recipients. The irradiation was accomplished by means of a General Electric OX-220 Maximar industrial unit. Food was withheld for 12 hours and the dogs were placed under general anesthesia with pentobarbital sodium. Active pups were tranquilized by means of intramuscular injections of 50 mg. of promazine hydrochloride. The anesthetized dogs were placed in a cylindrical container with an internal diameter of 28 cm. and a height of 9 cm. The container was placed directly under the porthole at a distance that resulted in a filament-to-body midline distance of 50 cm., which was maintained on all animals.[10] The animals were turned over after half the exposure time in order to achieve a uniform radiation effect throughout the body. The unit was operated at 220 kv. and

* Sterane, produced by Chas. Pfizer & Co., Inc., New York, N.Y.

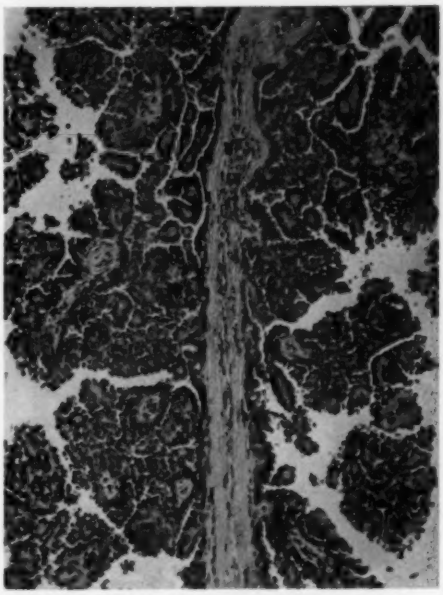

Fig. 1a—Ovarian adenocarcinoma from donor A1742; an extremely loose, papillary arrangement of tumor cells placed on a scant fibrous stroma. H & E stain; x 233.

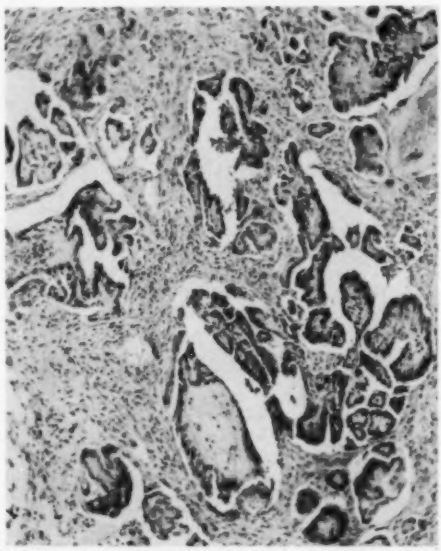

Fig. 1b—Transplanted ovarian adenocarcinoma in the inguinal subcutis of recipient (A1702). The vigorous, papillary tumor tissue is invading the corium and subcutis, in which there is only a slight inflammatory reaction. H & E stain; x 170.

9 ma. To cut out the soft rays, a 1-mm. aluminum filter and a 0.5-mm. copper filter were inserted at the porthole. At this setting, the calculated output was found to be approximately 33 r per minute as measured in air at a distance of 50 cm. Dosage calibrations were made by an X-ray physicist.

TABLE 2—Attempts to Transplant a Venereal Tumor and an Ovarian Adenocarcinoma in Irradiated and Prednisolone-Treated Dogs (Experiment A)

Dog No.	Age (days)	Radiation (r)	Prednisolone (mg.)	Necropsy day*	Growth
A1340	75	600	20	13	Negative
A1341	75	600	20	Alive	Negative
A1701	40	600	20	Alive	Negative
A1702	40	600	20	15	Ovarian adenocarcinoma
A1703	40	600	20	24	Negative
A1704	40	600	20	7	Negative

* Interim between irradiation and necropsy.

After termination of the radiation, the dogs were returned to their cages and the injection of tumor tissue was accomplished after different time intervals during the following 1 to 7 days. The inoculated dogs were examined twice daily, and in 2 irradiated groups the pulse and body temperature were recorded daily. In order to evaluate the biologic effects produced by the calculated dose of radiation in the exposed dogs, the total white blood cell count, the platelet count (direct method), the hemoglobin concentration, and the differential white blood cell count were determined at short intervals during the course of this study.

In order to avoid infections in the irradiated and cortisone-treated dogs, only clinically healthy dogs, born and raised under the authors' supervision, were used. Autoclaved instruments were used to handle the implants, and the recipients' skin was thoroughly scrubbed with a skin disinfectant prior to all injections. Daily administration of penicillin and streptomycin, either separately or in combination, were given to the irradiated dogs. Complete necropsies were carried out on all dogs and histopathologic studies were made on tissues fixed in 10% buffered formalin. The stains or methods used were hematoxylin and eosin, Masson's trichrome, McManus' periodic acid-Schiff, and Giemsa. Serial sections of the inoculation sites were made as needed. Sixty-seven implants from 17 neoplasms were made into 48 dogs (Table 1), but only the 3 experiments (A, B, and C) in which successful growth occurred will be described in detail.

Experiment A.—Twenty-four hours after 6 dogs were exposed to 600 r of total body irradiation (Table 2), 1 ml. of minced tumor tissue from a venereal tumor (A1692, in penicillin and saline) was given subcutaneously in the left groin and 1 ml. was given intraperitoneally to all 6 dogs. The recipients were given 250 mg. of streptomycin daily and 20 mg. of prednisolone every other day, beginning on the day of tumor inoculation. Seven days after irradiation, 4 ml. of a minced tissue suspension (in saline solution and penicillin) from an ovarian adenocarcinoma (Fig. 1a) of donor A1742 was injected subcutaneously into the right groins of dogs A1341, A1702, and A1703. The 3 dogs were selected because the first transplants from A1692 had remained stationary, probably indicating unsuccessful growth.

Experiment B.—Six dogs from 2 different litters were subjected to total body irradiation while under anesthesia (Table 3). B898 and B899 were given 465 r, while the remaining 4 were given 600 r. Prednisolone (10 mg.) was administered daily to B898 and B900 from the day of irradiation until death, and all dogs were given 0.5 ml. of a penicillin-streptomycin mixture daily. Three different implantations were made. Two hours after irradiation, 5 ml. of a seminoma (from B424) suspension in nutrient medium was injected subcutaneously in the right groin and 4 ml. of a mammary tumor (from B549) suspension in nutrient medium (Fig. 2a) was injected into the left groin of each of the 6 dogs. Seven days later, 2 ml. of a 7-day-old tissue culture growth from the mammary tumor of B549 was subcutaneously injected anterior to the umbilici of B898 and B900.

TABLE 3—Fate of a Seminoma, a Mixed Mammary Tumor and a Tissue Culture of the Latter, Implanted in Irradiated and Prednisolone-Treated Dogs (Experiment B)

Dog No.	Age (days)	Radiation (r)	Prednisolone (mg.)	Necropsy day	Active growth
B896	50	600	None	3	Negative
B897	50	600	None	10	Negative
B898	50	465	10	12	Mammary tumor
B899	50	465	None	Alive	Negative
B900	26	600	10	10	Mammary tumor
B901	26	600	None	10	Mammary tumor

Experiment C.—Twenty-four hours after irradiation with 500 r, 5 ml. of minced osteosarcoma tissue (Fig. 3a) in nutrient medium was injected subcutaneously into the umbilicus of each of 4

TABLE 4—Fate of an Osteosarcoma Implanted in 4 Irradiated and Prednisolone-Treated Dogs (Experiment C)

Dog No.	Age (days)	Radiation (r)	Prednisolone (mg.)	Necropsy day	Result
B902	47	500	10	10	Transitory growth
B903	47	500	None	10	Transitory growth
A1064	385	500	10	13	Transitory growth
A1705	244	500	None	13	Active growth

dogs (Table 4). All were given 0.5 ml. of streptomycin daily, and 2 were given 10 mg. of prednisolone daily.

Results

The benefits that the tumor implants derived from the 2 hormone preparations in this study are difficult to evaluate because of the small number of animals in which there was successful growth. However, under the conditions present, the doses of adrenal corticoids were not sufficient to allow growth of the transplants in nonirradiated dogs. The necropsy of dogs given prednisolone for 2 weeks or more revealed atrophy of the adrenal cortex.

Experiment A.—The initial subcutaneous swelling, after the inoculation of tumor cells, lasted for 12 to 24 hours. This was followed by a gradual increase in volume and firmness, reaching the maximum size in 8 or 9 days. The recipients either died from radiation sickness or were killed at the following intervals (Table 2): A1704 on the 7th, A1340 on the 13th, A1702 on the 15th, and A1703 on the 24th postirradiation day. Dog A1702 had a positive "take" with an actively growing ovarian adenocarcinoma surrounded by necrosis and hemorrhage (Fig. 1b). The other transplants had various stages of necrosis, resorption, and scarring. Two dogs, A1341 and A1701, failed to develop any tumor growth during the following 18 months.

Experiment B.—All dogs except B899 died from radiation sickness on the 3rd to the 12th day postirradiation. Three dogs, B898, B900, and B901, developed small, firm, oblong swellings in the left groin measuring 6 by 4, 10 by 5, and 8 by 6 mm., respectively, at the 7th day after implantation, indicating active growth of the mixed mammary tumor (Table 3). Histopathologic examination of the 3 recipients

TABLE 5—Summary of Data from 5 Dogs with Successful Growth of Transplants

Dog No.	Age (days)	Radiation (r)	Prednisolone (mg.)	Neoplasm
B898	50	465	10	Mixed mammary tumor
A1705	244	500	None	Osteosarcoma
B900	26	600	10	Mixed mammary tumor
B901	26	600	None	Mixed mammary tumor
A1702	40	600	20	Ovarian adenocarcinoma

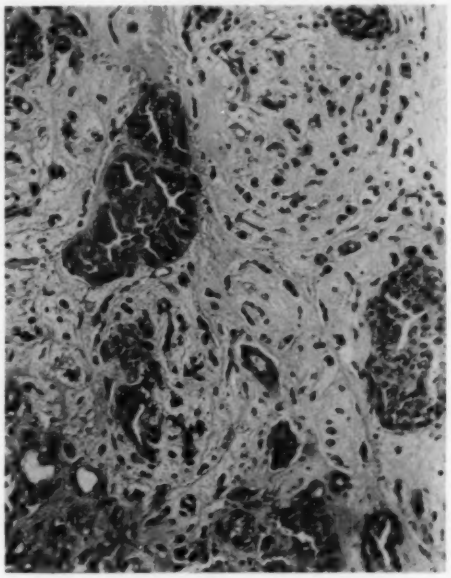

Fig. 2a—Mixed mammary tumor from donor (B549); neoplastic mammary ducts surrounded by a mucinous and chondroid stroma. H & E stain; x 240.

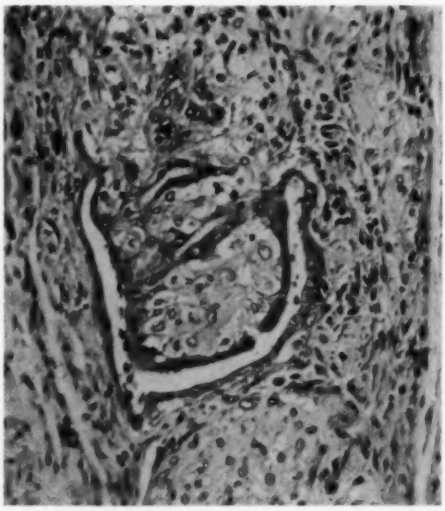

Fig. 2b—Transplanted mixed mammary tumor from the subcutis of recipient (B900); notice neoplastic duct structure surrounded by connective tissue undergoing mucinous and chondroid metaplasia. H & E stain; x 360.

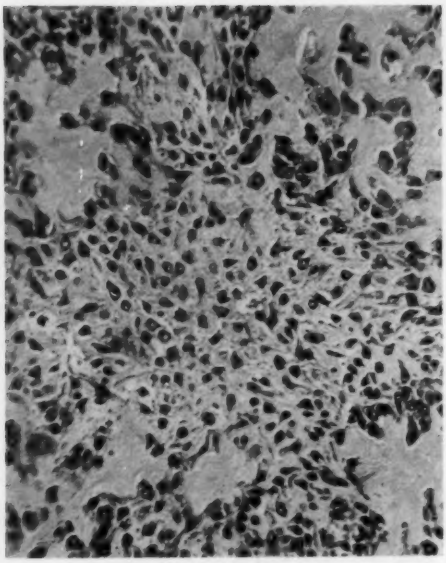

Fig 3a—Osteosarcoma from the radius of donor (B989); notice immature, fusiform osteoblasts with large ovoid nuclei and prominent nucleoli. Osteoid matrix is being formed. H & E stain; x 360.

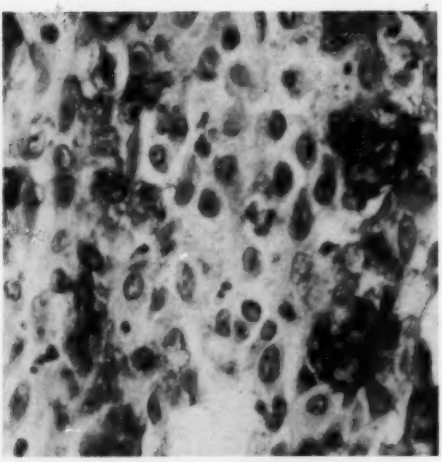

Fig. 3b—Transplanted osteosarcoma from the inguinal subcutis of the recipient dog (A1705), neoplastic ovoid nuclei of the osteoblasts with swollen nucleoli and one Y-shaped mitotic figure are depicted. Many mineralized bone spicules are seen. H & E stain; x 1057.

revealed almost identical findings. Neoplastic tubular structures of large epithelial cells with vesicular nuclei were surrounded by a light bluish, slightly vesiculated, myxoid stroma which closely simulated the donor's tumor. Considerable fibrosis surrounded the areas of viable tumor growth (Fig. 2b). The right groin, containing the seminoma transplant, developed a transitory swelling which disappeared after 24 hours, indicating failure of growth. Bilateral adrenal cortical atrophy was present in both dogs given prednisolone.

Experiment C.—The 2 young dogs (B902 and B903) died on the 10th day, and the 2 older dogs died on the 13th post-irradiation day from radiation sickness with pulmonary edema and multiple hemorrhages. Dog A1705 had actively growing osteoblasts (Fig. 3b) with mitotic figures, while the other 3 dogs only had evidence of early transitory growth, which subsequently ceased and became necrotic. No difference was grossly evident between the positive and negative implants. They all appeared as white, lobulated, ovoid nodules surrounded by red zones of hemorrhage and fibrin, which gradually were transformed into fibrous tissue (Fig. 4). No beneficial effect on tumor cell growth was seen in the prednisolone-treated dogs as compared with the non-treated dogs.

The necropsy findings in dogs dying from radiation sickness after exposure to 465 r or more will be described elsewhere.[24] At this level of radiation, approximately 50% of the exposed dogs died within 30 days. The pathologic changes differed only in degree from those observed by Mulligan [22] and by Gleiser,[7,8,9,10] who used high voltage radiation in dogs, and from those described in other species of animals by Ellinger.[4] The changes were pulmonary edema; diffuse hemorrhages and necrosis of the lymph nodes, spleen, tonsils, thymus, Peyer's patches, and bone marrow; and focal hemorrhages of the gastrointestinal mucosa. Approximately one week following irradiation, the lymph nodes and bone marrow were wholly depleted of lymphoid and myeloid cells and contained only a syncytium of reticulum cells surrounded by erythrocytes. The white blood cell count and the platelet count were the most accurate indicators of radiation damage. The hemoglobin content and the dif-

Fig. 4—Gross photograph of an unsuccessful osteosarcoma transplant; transitory growth only, in the inguinal subcutis of the recipient pup (B903). The lobulated, white, gritty tumor is surrounded by fibrin and hemorrhage.

ferential count were found to be less useful. The postirradiation decrease in hemoglobin occurred many days after leukocyte and thrombocyte numbers had decreased, which made hemoglobin determination of less practical value. Differential counts were made on all blood samples, but further work would be necessary to determine fully their prognostic value. Both the leukocyte and thrombocyte counts decreased at a rapid rate from the day of irradiation. In dogs surviving a dose of 300 r, the circulating leukocytes dropped from a preirradiation level of 12,000 to 19,000 per cubic millimeter to 1,000 per cubic millimeter on the 11th to 16th day. After the low point was reached, the leukocyte count began to rise, probably indicating regeneration from surviving reticuloendothelial stem cells. The thrombocytes

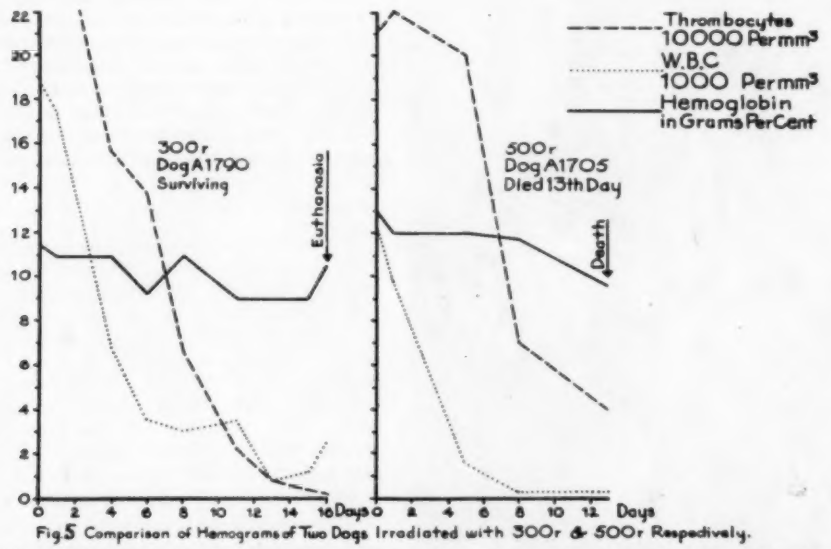
Thrombocytes 10000 Per mm³
W.B.C 1000 Per mm³
Hemoglobin in Grams Per Cent
300r Dog A1790 Surviving
Euthanasia
500r Dog A1705 Died 13th Day
Death
Days
Fig.5 Comparison of Hemograms of Two Dogs Irradiated with 300r & 500r Respectively.

Fig. 5—The ordinate of this graph indicates units of hemoglobin, thrombocytes, and leukocytes. The abscissa indicates time following irradiation. The decline of the thrombocyte count in both dogs occurred later than that of the leukocyte count. Notice the rise of hemoglobin and leukocytes occurring in the recovering dog (A1790).

dropped from normal levels of 200 to 300,000 per cubic millimeter to about 5,000 per cubic millimeter at the 15th to 20th day after irradiation. The low point usually occurred a few days later than that of the leukocytes (Fig. 5). In dogs given larger doses, deaths occurred from the 3rd to the 16th day, with the majority between 10 and 13 days. The leukocytes and thrombocytes declined precipitously until the time of death, as seen in dog A1705, given 500 r (Fig. 5). Microscopically, there was a striking similarity between the morphology of the original tumor in the donor and the transplanted tumor in the subcutis of the recipient (Fig. 1–3). By studying unsuccessful implants at various time intervals, it was seen that some tumors never had active proliferation and enlargement, but that they became necrotic immediately after transfer to the recipients. The 2 seminomas (B424 and B697) that were transplanted into 8 dogs behaved in this manner. From 3 tumors (B424, B723, and B1480), explants grew well in tissue cultures (Fig. 6), while parallel transplants failed to grow in the recipient dogs. In other tumors, there was evidence of active growth with progressive enlargement of the original implant for several days before they ceased to grow, usually one week after implantation. This occurred in 3 of the osteosarcoma transplants (Tables 1, 4). These transplants underwent degenerative changes and atrophy with scarring of the surrounding subcutis, probably indicating regeneration of the host's defense reactions. Even in dogs where complete lymphoid and myeloid depletion after irradiation was evident on all sections examined, regression of the implant still occurred. This may be explained in part by either lack of growth ability of the implant or by survival and regeneration of the reticuloendothelial cells somewhere in the body of the recipient.

Fig. 6—Tumor cells from a 3-day-old tissue culture of a chondrosarcoma from donor B723. H & E stain; x 360.

Bacterial infection of the implants in the irradiated and cortisone-treated dogs was avoided satisfactorily; only 1 of 67 implants developed evidence of infection with subsequent abscess formation. Likewise, systemic bacterial infections were prevented by the daily penicillin and streptomycin injections. It was not possible to completely prevent nonbacterial diseases in the inoculated dogs. Three dogs, A985, A986, and A1062, developed distemper and hepatitis despite previous injections of antiserum against both diseases and vaccination against distemper. A 4th dog, B1522, succumbed to toxoplasmosis on the 100th postirradiation day. This dog also was given cortisone for 21 days beginning on the day of irradiation.

Discussion

In autotransplantation and isohomologous transplantation, successful growth can be obtained without pretreatment of the recipient. In the former, a piece of tissue or an organ is transplanted from one site of the body to another as a part of an experimental procedure. In isohomologous transplantation, the implant is made from one animal to another of the same inbred strain; this has been successful in mice and rats. Transfer of tissue from a donor of one species to a recipient of another species is known as heterologous transplantation, and success in adults has usually required pretreatment with x-rays or compounds such as cortisone or nitrogen mustard. This is also true when tissue is trans-

planted between 2 individuals of the same species, but of different genotypes. Successful heterologous transplantation without pretreatment can be made, however, into certain tissues of the recipient where antibody formation is known to be very low, like brain, testes, and the anterior chambers of the eyes, as shown by Green [12] and Towbin.[32] A few tumors are known to have been grown successfully by transfer of tumor cells from one animal to the subcutis of another unrelated adult member of the same species, *i.e.*, the canine venereal tumor and the Brown-Pearce tumor of rabbits.[27]

All factors necessary for the successful growth of an implant in the recipient's tissues are not known. Many uncontrollable variables are present in both the implant and the recipient. It is apparent that great variability exists in the implanted cells as to type of neoplasm, its state of nutrition at the time of removal, its cell division rate, its stroma content, and many other factors. As these cannot be controlled, it is important that no unnecessary variables are introduced. The implant was handled in the same manner in each transplantation attempt with a constant time interval between removal and implantation. The block of tumor tissue to be used for transplants was selected from a deep part of the donor's tumor, to exclude surface contamination, and from an area which appeared healthy, having no evidence of hemorrhage and necrosis. Microscopic studies of the implantation sites showed that the vast majority of the implanted cells undergo necrosis, possibly as the result of ischemia and defense reactions of the host. The presence of necrosis may not be an entirely undesirable feature as cell compounds from the dead tumor cells may serve as nutrition for more vigorous, surviving cells, and the dead tissue may also enhance the vascularization of the implant. The number of tumor cells implanted is not the deciding factor for successful growth, as transplantation has been achieved by inoculation of a single cell by Furth and Kahn [6] in mouse leukemia and by Hauschka [13] in 2 other experimental tumors. The success or failure of an implant depends upon the ability of a few surviving cells to multiply and penetrate the immunologic and morphologic barriers of the host.

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SUMMARIO IN INTERLINGUA

Transplantation Homologe de Neoplasmas Canin

Sexanta-septe implantas preparate ab neoplasmas canin esseva implantate in 48 canes. Esseva considerate in iste studio duo factores facilitante le crescentia del transplantas, i.e. (1) tractar le recipientes con corticosteroides e (2) tractar los con irradiatnon del corpore total. Duo corticosteroides esseva usate: Cortisona in un dose de 25 mg per die in canettos de 500 g de peso e prednisolona in doses de 5 e 10 mg per die o de 20 mg omne secunde die in canettos dislactate. Trenta-un canes esseva irradiate con doses de inter 170 e 600 r.

In 5 canes, successo del transplantation esseva obtenite. Illos omnes habeva essite irradiate, 1 can con 465, 1 con 500, e 3 con 600 r. In plus, 2 habeva essite tractate con 10 mg de prednisolona per die e 1 con 20 mg de prednisolona omne secunde die. Le tumores a successo de transplantation esseva un oseteosarcoma, un mixte tumor mammari, e un adenocarcinoma ovarian. Le characteristicas histologic del transplantate tumor in le subcute del recipientes esseva indistinguibile ab illos del tumor in le donator.

It es permittite concluder que depression del systema reticuloendothelial per hormones adrenocortical e irradiation del corpore total esseva necessari pro le successose crescentia del transplantas canin usque nunc essayate. Tractamento adrenocorticosteroide sol e irradiation del corpore total in doses de minus que 465 r esseva insufficiente pro permitter le crescentia del transplantas, sed le combination del duo mesuras resultava in un certe grado de successo. Le methodo le plus efficace pareva esser radiation a alte dosage, in le region de magnitudes ab 465 ad 600 r.

Pulmonary Adenomatosis of Sheep—Metastasizing Bronchiolar Tumors

Alberto Cuba-Caparo, M.D.; Elmo de la Vega, D.V.M.; Marcos Copaira, D.V.M.

SUMMARY

During an investigation to determine the histogenesis of pulmonary adenomatosis in sheep from the Central Sierra of Peru, the lungs of 10,600 apparently normal sheep were examined and 6 affected sheep were observed. Of 60 cases detected grossly and microscopically, 22 involved incipient lesions; 38, advanced lesions.

In this investigation, 3 cases of metastases of the mediastinal lymph nodes were found. Some histologic aspects attested to the bronchiolar origin of the epithelial proliferation.

INTRODUCTION

In 1945 there was reported [4] in Junin, Peru, a sheep disease with morphologic characteristics comparable to those of chronic progressive pneumonia described by Marsh in Montana,[18] to jagziekte of South African sheep,[26] and to epizootic pulmonary adenomatosis described by Dungal in Iceland.[6] Pulmonary adenomatosis has also been described in horses,[32] chinchillas,[13], cats,[23], mice,[33] guinea pigs,[11] and cattle.[21, 22, 27]

Pulmonary adenomatosis in man was described by Helly [12] in 1907. Since that time, many similar lesions have been reported extensively in the medical literature. In a review of this subject, Laipply *et al.*[15] mentioned the large number of terms which have been used in referring to this disorder. They made a study of its morphologic features and clinical course and reported 8 personally observed cases. It was their conclusion that the lesions of alveolar carcinoma and pulmonary adenomatosis were related and were of bronchiolar origin.

Received for publication Aug. 5, 1960.

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The authors thank Eng. R. Calle for the many facilities he made available for this investigation.

With the aim of establishing the histogenesis of pulmonary adenomatosis in sheep, and of reporting metastasizing forms of the disease, the present investigation was carried out.

The lungs of 10,600 apparently normal sheep killed at the National Frigorific, Lima, Peru, were examined with this purpose in view. The investigation included examination of the regional lymph nodes and other organs. Fifty-four examples of the condition were detected, 22 of which were classified as incipient lesions, and 32 were identified as advanced lesions.

Materials and Methods

In all, 60 cases of pulmonary adenomatosis were investigated histopathologically. As indicated, 22 were classified as incipient forms and 32 as advanced stages on the basis of the size of the lesions and their diffusion into other pulmonary lobes. Those limited to one lobe, with a diameter not larger than 2 cm., were considered incipient. Larger lesions, located in one or more lobes, were considered advanced. In the 54 cases from the National Frigorific, the lungs, trachea, bronchial lymph nodes, kidneys, heart, and liver were examined. Six additional sheep were procured from a farm in the Central Sierra—4,200 meters above sea level. These animals were observed clinically for a period varying from 8 to 40 days. Advanced pulmonary adenomatosis was the clinical diagnosis in each of them. Two were Corriedales; the other 4 were crossed native Corriedale strains.

The sheep obtained at the National Frigorific were adult crossbred descendants of Corridale and Merino strains from different parts of the country. It was not possible to determine the exact place of their birth or their origin.

Observations

The gross and microscopic features of pulmonary adenomatosis described in this report represent those observed in the present 60 cases and in 15 cases previously reported by Cuba-Caparo.[4]

Gross Appearance.—The most striking feature was the presence of multiple, round, white or yellowish granular lesions of variable size. The smaller ones were barely noticeable grossly. The larger ones were 5 to 10 mm. in diameter and were easily

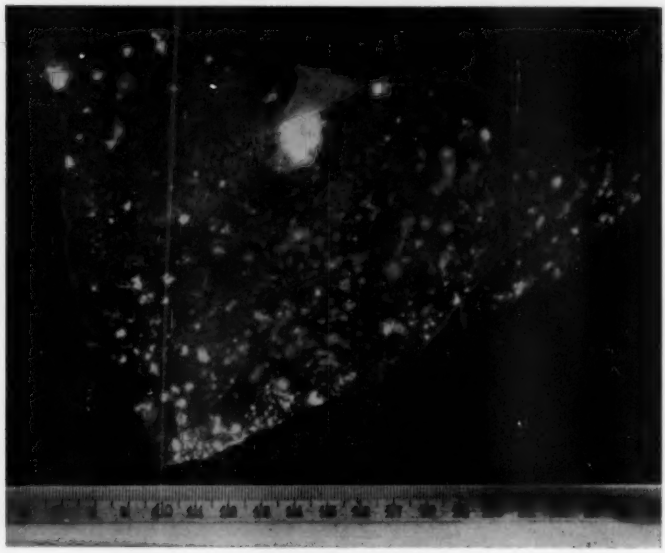

Fig. 1—Right lung viewed from the cut surface. Adenomatosis, nodular form.

detected. Both were considered to be the initial lesions of pulmonary adenomatosis which subsequently became confluent in some instances, thus increasing the bulk and consistency of the affected lung. The gross alterations were such as to give the lung a pneumonic appearance.

In some instances, the nodules remained isolated; in others, they became confluent. On this basis, 2 essentially different microscopic forms were distinguished: a nodular form in which isolated nodules predominated, and a pneumonic form in which confluent lesions predominated. There was also a 3rd form characterized by the presence of soft zones of necrosis and abscess formation.

Figures 1 and 2 show the nodular form characterized by small, multiple nodules. Figure 3 shows a more advanced lesion in

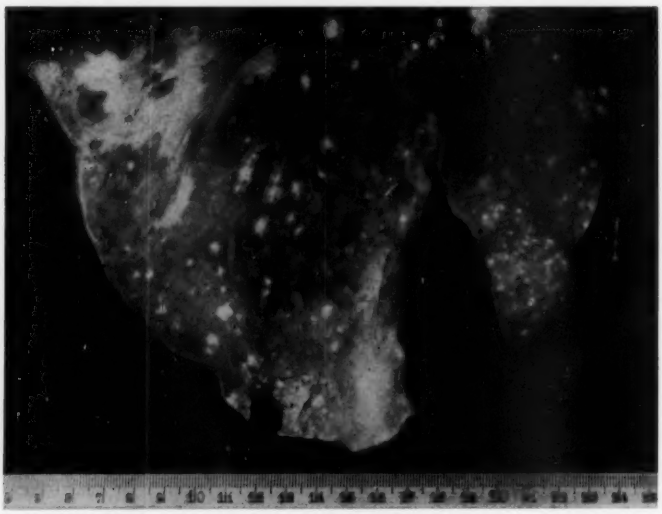

Fig. 2—Same as Figure 1 viewed from the exterior surface through the pleura.

which the greater part of the lung contains multiple, independent nodules. These nodules were gathered near the hilar region, became confluent, and assumed the appearance of hepatization (Fig. 4).

The nodular form was more frequently observed in the incipient stages of pulmonary adenomatosis. The confluent or pneumonic form was found in more advanced stages. The necrotizing form was characteristic of the terminal stages of the disease. It was encountered in animals that were not killed but died as a consequence of the progressive evolution of the disorder.

Lesions were observed with equal frequency in both lungs. Bilateral lesions were found in 24 of 60 sheep. In 18 cases, the right lung alone was affected; in an equal number, the left lung was affected. There was no preferential localization in any particular lobe, although in incipient forms the diaphragmatic lobes were commonly affected.

Even in the incipient forms, there was a nacreous thickening of the visceral pleura, in some cases 1 to 2 cm. thick. This thickening was more marked in the advanced lesions and was frequently associated with resistant adherence to the parietal pleura. In the pleural cavities, a variable amount of liquid was found; in some cases this was abundant, yellowish, and turbid. It contained coagulated fibrin in most instances.

An increase in the number and volume of the anterior mediastinal lymph nodes was observed in sheep with advanced infection, particularly in those with gray hepatization, bronchitis, or other inflammatory lesions. The size of the nodes was greater in those in which metastases were observed microscopically. A whitish color in the external as well as on the cut surface of the nodes was noticed. However, a correct gross diagnosis was made only in 1 of the 3 cases of metastases encountered. The gross features of lymphoid hyperplasia were such that they could not be distinguished from metastases.

Significant lesions were not observed in other organs.

Microscopic Observations.—In all cases, the most striking feature was the presence of cylindrical cells lining alveolar spaces and arranged in single layers. The epithelium contained an ovoid vesicular central nucleus with sparse chromatin and 1 or 2 nucleoli. The cytoplasm in most instances was compact (Fig. 5), but in some

Fig. 3—Right lung. Nodular and diffuse or pneumonic form of adenomatosis.

was frankly vacuolated (Fig. 6). These 2 features were occasionally evident in the same microscopic section. The vacuolated structure of the cytoplasm was due to the presence of mucin demonstrated by periodic acid-Schiff (PAS) staining. The free margins of the cells in some instances contained small spheres of secretion; in others, the free margin was ciliated.

Proliferation of the alveolar lining cells had the characteristics of neoplastic growth. Atypical nuclei were not found, however, and the normal alveolar architecture was retained. On the other hand, projecting papillary ingrowths into the alveolar spaces were frequently observed (Fig. 7). In other instances, the abnormal cells invaded respiratory ducts and terminal bronchioles. In the advanced lesions the proliferation of the alveolar lining cells was not diffuse at the margins. On the contrary, isolated zones of pulmonary parenchyma were affected and nodules were surrounded by alveoli with normal appearance (Fig. 8).

The cylindrical lining cells occasionally overlaid normal alveolar septums. At other times, however, the alveolar walls were markedly thickened by inflammatory infiltration of lymphoid cells or loose connective tissue. In 2 instances, mucous nodules similar to those described by Cowdry [2] and McFadyean [19] were observed. The incidence of lymphoid nodules was less frequent than indicated by the reports in the Montana form of chronic progressive pneumonia.

The content of the affected alveolar spaces was variable: cylindrical cells, round cells with great cytoplasmatic mass (alveolar cells), neutrophils, and lymphocytes were observed. In the advanced forms, this desquamation was more intense and was accompanied by peribronchial inflammatory infiltration, the formation of lymphoid nodules, and catarrhal alveolitis. Generally, the inflammatory component was regularly present. Even in the parenchyma surrounding the nodules, catarrhal alveolitis was observed. In the advanced forms of pulmonary adenomatosis, the inflammatory infiltration was marked and, in sections made in the region of hepatization, there was fibrinous exudate containing abundant neutrophilic leukocytes. In those sheep dead from progressive evolution of the disease, extensive zones of necrosis and abscess formation were found. Invasion of epithelial cells into blood vessels was not observed.

The visceral pleura was regularly thick-

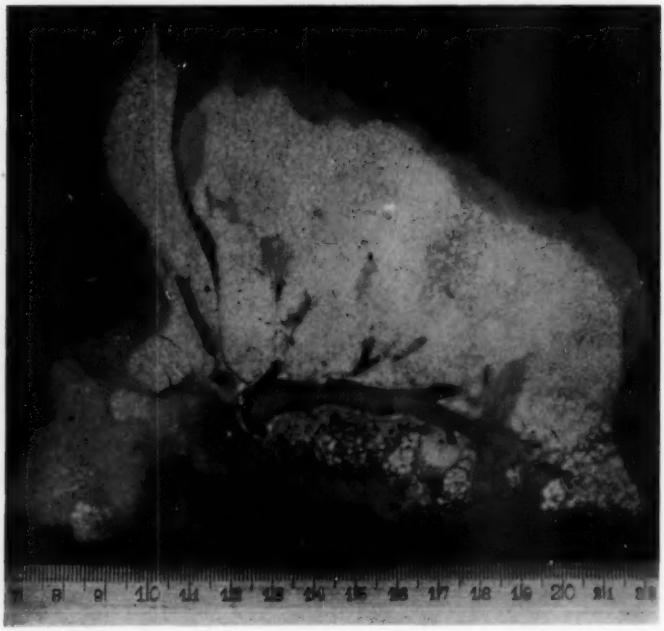

Fig. 4—Right lung. Diffuse or pneumonic form of adenomatosis.

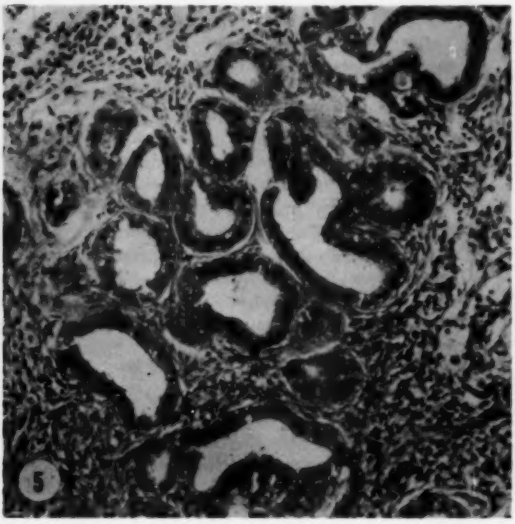

Fig. 5—Cavities lined with cylindrical cells of compact cytoplasm. H & E stain; x 160.

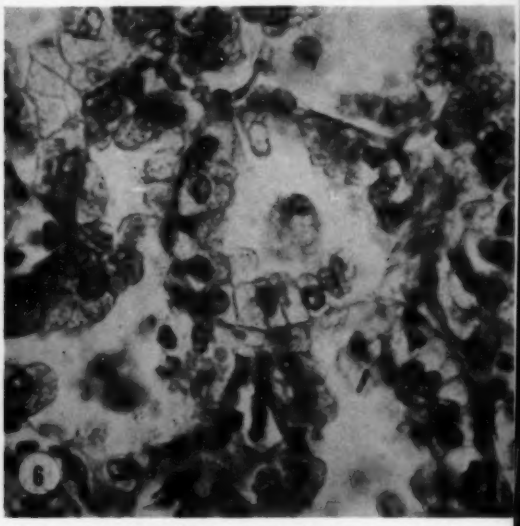

Fig. 6—Cavities lined with cylindrical cells of vacuolar cytoplasm. H & E stain; x 690.

ened. In the incipient forms, the thickening was limited to the regions which contained subpleural nodules. In the more advanced stages it was diffuse, covered large lobar areas, and was accompanied by an inflammatory infiltration composed of lymphocytes, neutrophils, and newly formed capillaries.

Except for the lymph nodes, there were no significant lesions in other organs. The liver, heart, and trachea were examined in 54 cases from the National Frigorific. In the 6 farm cases, the necropsies were complete except for the nervous system.

Incipient Lesions and Their Probable Histogenesis (22 Cases)

Gross Examination.—As indicated, these lesions were observed in 2 forms: a nodular variety composed of small independent white or yellow nodules, 1 to 2 mm. in diameter, and a diffuse or pneumonic form composed of small foci of hepatization with compact and homogeneous structure. The lesions were located at the periphery of the lungs and were subpleural in distribution.

The distribution of the lesions may be seen (Table 1). The right and left lungs were equally affected but the lesions were located predominantly in the diaphragmatic lobes.

Microscopic Findings.—The features of the incipient lesions were essentially the same as described in the first part of this report. In cases in which the nodular lesions were not confluent or at the periphery of the areas of consolidation, epithelial proliferation was marked in relation to the terminal bronchioles. Occasionally, a bronchiole was seen in the center of the nodules. In some longitudinal sections, there was continuity of bronchiolar epithelium with the intra-alveolar epithelial proliferation (Fig. 9).

The PAS staining of alveolar epithelium was not a constant feature. In some cases, it was not found at all. In others, it was variable in different fields in the same section. In some lesions, the cylindrical cell lining resembled bronchial epithelium. In others, acinar structure was observed. Cilia were detected in a few cases. They were noticed in the papillary exerescences which developed in the bronchioles, but did not

TABLE 1—Distribution of the Adenomatosis Lesions in the Different Pulmonary Lobes

Lung	Total specimens	Apical lobes	Cardiac and medial lobes	Diaphragmatic lobes
Right lung	11	2	2	7
Left lung	11	3	1	7

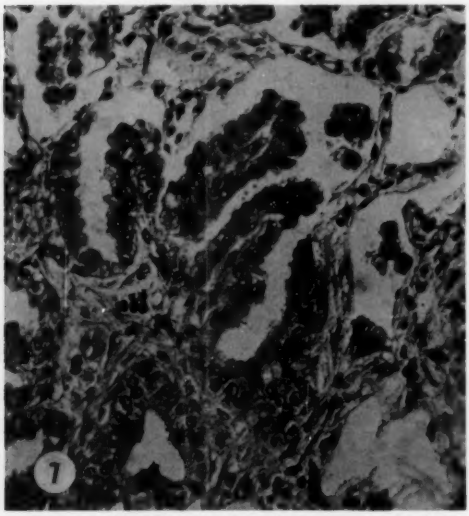

Fig. 7—Alveolar cavities lined with cylindrical cells and protecting papillae. H & E stain; x 280.

appear in the lining of the alveolar spaces. This variation was probably related to the degree of differentiation attained by the epithelial elements. Cowdry [2] and Cowdry and Marsh,[3] studying jagsiekte and the Montana chronic progressive pneumonia, found thickening of alveolar walls. We also observed this, but not to as marked a degree. In some regions of the lungs, it was accompanied by epithelial proliferation. In others, it appeared in alveolar septums of otherwise normal appearance. It is our belief that thickening of the interalveolar substance constitutes a nonspecific lesion of secondary nature, since it occurs in a number of other unrelated conditions (bronchopneumonia, caseous lymphadenitis, actinomycosis) in sheep native to the portions of the country where pulmonary adenomatosis has not been observed.

Metastases to the Mediastinal Lymph Nodes

The capacity for metastases in pulmonary adenomatosis of Peruvian sheep was demonstrated by Paredes,[24] who, in a series of 20 necropsies, found 4 instances of metastases to the mediastinal lymph nodes. Three instances were encountered in the present group of cases, 2 occurred in the terminal stages of the disorder, and 1 was encountered in an earlier stage.

Discussion

An analysis of the various theories proposed in connection with the existence of an epithelial lining of pulmonary alveoli, and the relationship of this lining to inflamma-

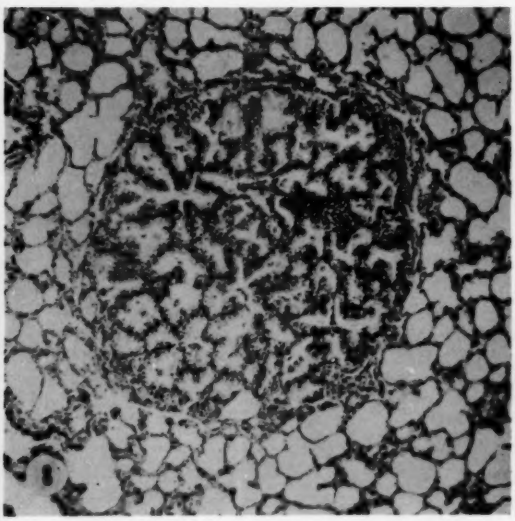

Fig. 8—Adenomatous nodule surrounded by pulmonary parenchyma of normal appearance. H & E stain; x 160.

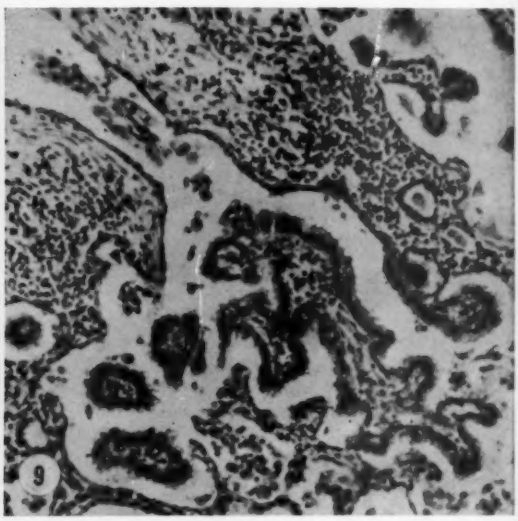

Fig. 9—Terminal bronchiole. The cylindrical epithelial lining is continuous with the epithelial proliferation of the alveolar cavities. H & E stain; x 160.

tory or neoplastic processes, is not attempted. A careful evaluation of these hypotheses and the related bibliography may be found in the reports of Laipply,[15] Swan,[31] Miller,[20] Loosli,[17] Lang,[14] and Geever.[9]

In accordance with the concepts of Laipply [15] and Liebow,[16] it is our belief that the epithelial component in pulmonary adenomatosis is of bronchiolar origin. In the 22 earlier cases we examined, continuity between the cells lining the alveoli and bronchiolar epithelium were manifest. Moreover, intrabronchiolar papillas were observed to originate from bronchiolar epithelium. The existence of mucin and cilia, although not constant features, further supports this interpretation. Cilia and mucin secretion are accepted characteristics of the bronchiolar neoplasm. Additional support is gained from the fact that the pulmonary proliferation is multicentric, subpleural in location and, though having continuity with bronchiolar epithelium, is unrelated to the epithelium of major bronchi.

De Kock [5] studying the African form of pulmonary adenomatosis of sheep (jagsiekte) considered the epithelium to be derived from the epithelium of the alveolar walls and bronchioles. The neoplastic nature of pulmonary adenomatosis in sheep was demonstrated for the first time by Aynaud [1] in 1926. He found one instance of metastasis in a bronchiolar lymph node. Later, Dungal examined Aynaud's slides and confirmed his interpretation. In 1953, Paredes [24] found mediastinal lymph node metastasis in 4 cases of pulmonary adenomatosis. As far as is known, no metastasis has been described in pulmonary adenomatosis of sheep in Germany,[25] Iceland,[28] England,[19] South Africa,[30] or North America.[18]

TABLE 2—Clinical Examination, Gross and Histologic Findings in 3 Cases of Pulmonary Adenomatosis with Metastases in Lymph Nodes

Clinical examination	Necropsy findings	Histologic findings
CASE 1		
A Corriedale ram, imported from Chile, 7 yr. old, killed after living 6 years at 4,200 meters above sea level. Peculiar position with lowered head. Extreme difficulty in walking. Emaciated. Temperature varying between 39 and 40 C. Cyanosis. Tubarian puff, pleural friction râles and crepitant râles in both lungs.	Abundant yellowish turbid fluid in the pericardial sac and both pleural cavities. The lungs were equal in size, weighed 1,880 Gm. (normal 400 Gm.). Cardiac, medial, and diaphragmatic lobes of the right lung completely solidified. Cardiac and medial lobes contained 3 cavities, 5, 4, and 3 cm. in diameter filled with green exudate. Complete hepatization of left lung. Lymph nodes were enlarged and edematous. On the right side the bronchial lymph node contained a yellowish nodule 3 mm. in diameter.	Lungs—Adenomatosis; exudative pneumonia; abscess. Lymph nodes—Metastases were found in 7 of 37 lymph nodes: 2 anterior mediastinal nodes of the right side, 1 anterior mediastinal node of the left side, 3 paratracheal nodes, and 1 unidentified lymph node. In 2 cases, replacement of lymphatic tissue by neoplastic cells was complete. In some cavities papillary ingrowths were noticed (Fig. 10 and 11).
CASE 2		
A halfbreed Corriedale, born at 4,200 meters above sea level, was slaughtered at the age of 1 yr. Peculiar position with lowered head. Body temperature between 38.5 and 39.8 C. Moist and dry râles in both lungs. Abundant serous secretion from the nose.	Small amount of yellowish fluid in both pleural cavities. Adhesions in both diaphragmatic lobes. Lungs were increased in size, weighing together 1,485 Gm. Extensive areas of hepatization in all lobes and multiple nodules 1 to 2 mm. in diameter in the right diaphragmatic lobe. 2 cm. cavity was filled with purulent exudate in left medial lobe. Lymph nodes were slightly increased in size. No suspicious formations of metastases.	Lungs—Adenomatosis; abscess. Lymph nodes—1 of 20 anterior mediastinal lymph nodes had a small metastatic nodule 330 μ in diameter.
CASE 3		
From the National Frigorific. It was not possible to find out the origin and clinical data of this animal.	Multiple nodules, 1 to 3 cm. in diameter in both lungs. Slight increase in size of the lymph nodes. No suspicious formation of metastases.	Lungs—Adenomatosis. Lymph nodes—1 of 10 dorsal lymph nodes, neear the tracheal bifurcation, had a metastatic node, 1 cm. in diameter.

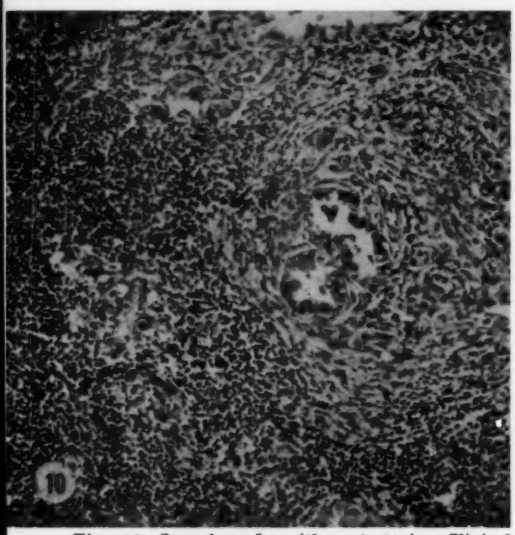

Fig. 10—Lymph node with metastasis. Clinical case No. 1. Photomicrograph shows epithelial cells lining cavities. H & E stain; x 100.

The frankly neoplastic nature of the metastases may be observed (Fig. 10 and 11). Although the incidence of metastases in our cases was comparatively low, it should be borne in mind that all but 6 of 60 animals were apparently healthy.

All of the animals observed by Paredes were in the last stages of the disease. It is probable that thorough examination of sheep with advanced lesions will show a higher incidence of metastases. Gross examination alone may not suffice since metastases were detected grossly in only 1 of our 3 cases.

Paredes personally advised us that he was able to detect metastases by gross examination of advanced cases because of the greater size of the nodes and the presence of foci of necrosis.

A comparative study of the lung sections from sheep with adenomatosis, with and without metastases, indicated that there were no fundamental differences in the histologic appearance. The characteristics of the nuclei and the epithelial pattern were the same in both. The alveolar architecture was retained. There was no atypical cellular pattern and neither infiltration nor invasion of blood vessels occurred.

A summary of Paredes' and our own observations indicates that pulmonary adenomatosis of Peruvian sheep affects both young and adult animals. We observed the disorder in lambs 3 to 4 months old. There is no evidence that this condition has an age predisposition. Paredes pointed out, however, that there is a greater incidence in animals dwelling at high altitude. Sheep

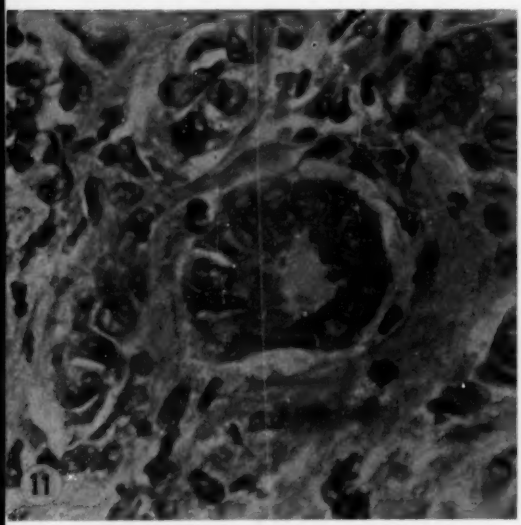

Fig. 11—Zone of epithelial cells of Figure 10 showing cavity lined with enlarged cylindrical cells. H & E stain; x 690.

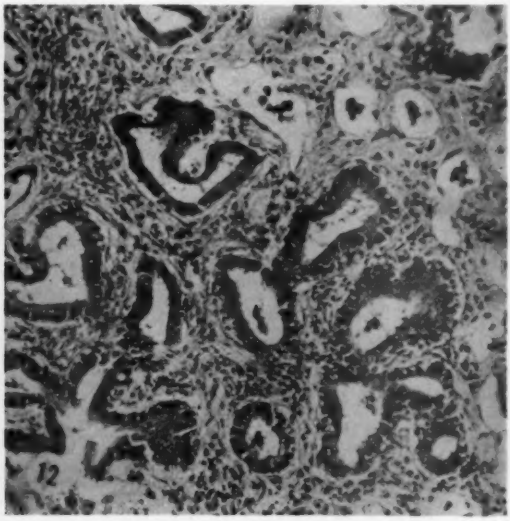

Fig. 12—Pulmonary adenomatosis of the goat. Zone showing cavities lined with cylindrical cells of compact cytoplasm similar to the one observed in Figure 5. H & E stain; x 160.

of all breeds may contract the disease. The course of the disorder is insidious and progressive. The duration cannot be determined. It seems to progress more rapidly in younger animals and results in death in a few months.

The nutritional state is not affected in the early stages of the disease but, in cases of prolonged duration, there is considerable emaciation. Body temperature is normal in the early stages of the disease; terminal hyperthermia is seen, with the occurrence of additional inflammatory lesions such as fibrinous pneumonia and pulmonary abscess formation.

The characteristic signs are: a tendency to keep the head in a lowered position, dyspnea, cough, and abundant nasal exudate.

Percussion and auscultation of the thorax reveals areas of consolidation, puffs, stertors, and pleural râles.

There is no close relationship between the incidence of the disease and the season of the year, although the mortality is greater in June and July. These winter months in the Sierra are characterized by variations of temperature from 20 C. at noon to −15 C. at night. There is no rain. The greater mortality in this season is due to secondary infections, bronchopneumonia being the most frequent. According to Paredes, the incidence of the disease increases with the extent of the altitude where the flocks are raised. The incidence of the disease, identified at necropsy, is as high as 30%.

The most affected regions of Peru is in the Central Sierra, which is one of the nation's important sheep-raising regions. The altitude varies from 3,500 to 4,500 meters above sea level. The disease has not been observed or ever reported in the northern or southern regions.

Pulmonary adenomatosis has not been observed in horses and cattle in Peru, but recently was identified in 2 goats killed at the National Frigorific (Fig. 12). It was not possible to determine their habitat, but it is known that goats are raised on the coast. On the other hand, before they are killed, they are frequently penned with sheep brought from the Sierra. Gross and microscopic features of the lesions in the 2 goats were in every way similar to those observed in sheep. Metastasis to mediastinal lymph nodes was not observed.

Concerning the etiology of pulmonary adenomatosis in sheep, attempts to transmit the disease by contact, lung extracts, or filtrates have been done in the United States, Iceland, and Africa.[5-8, 29] Because adenomatosis in Peru has a high incidence and is predominantly neoplastic and metastasizing in nature, we are engaged in an epizootiologic study with special reference to environmental conditions.

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SUMMARIO IN INTERLINGUA

Adenomatosis Pulmonar in Oves—Metastasante Tumores Bronchiolar

In le curso de un investigation del histogenese de adenomatosis pulmonar in oves ab le Sierra Central de Peru, le pulmones de 10.600 apparentemente normal oves esseva examinate, e 6 afficite oves esseva observate. Inter 60 macro- e microscopicamente detegite casos, 22 monstrava lesiones incipiente e 38 monstrava lesiones avantiate.

In iste investigation, 3 casos de metastase de nodos lymphatic mediastinal esseva trovate. Certe aspectos histologic indicava un origine bronchiolar del proliferation epithelial.

Erythrocyte Survival Studies in Domestic and Bighorn Sheep, Using Glycine-2-C^{14}

J. J. Kaneko, D.V.M., Ph.D.; C. E. Cornelius, D.V.M., Ph.D.; W. P. Heuschele, D.V.M.

SUMMARY

Erythrocyte survival was studied in 4 domestic sheep and a bighorn sheep. The median survival times were as follows: crossbred white-faced lambs, 94 days and 118 days; a Southdown lamb, 64 days; a Karakul ewe, 130 days; and a bighorn sheep, 147 days.

INTRODUCTION

Estimation, using glycine-2-C^{14}, of the erythrocyte survival times in various zoo species of the family *Bovidae* has been previously reported.[3] The median survival times for the erythrocytes of a mule deer and springbok antelope were 95 and 80 days, respectively. The aoudad or barbary sheep was found to have 2 separate populations of erythrocytes with median survival times of 65 and 170 days, respectively. In the present investigation, these studies have been extended to include the bighorn sheep *(Ovis canadensis)* and domestic sheep of various breeding. The erythrocytes were labeled *in vivo* with glycine-2-C^{14} from which the label is incorporated into 8 of the 34 carbon atoms of the porphyrin moiety of hemoglobin. The median survival times $(t\,^1/_2)$ of the erythrocytes of the sheep were estimated from their survival probability functions, $p(t)$, where $p(t)$ is the probability that an erythrocyte will have a survival time greater than t.[3, 4]

Materials and Methods

Data pertaining to the experimental animals and the amounts of isotope injected intravenously are given (Table 1). The bighorn sheep and Karakul sheep were housed at the San Diego Zoo. Erythrocyte counts were performed weekly on all animals. Twenty-milliliter samples of venous blood were obtained weekly from all animals; hemin was isolated,[5, 6] combusted to CO_2,[8] and measured for radioactivity.[3] The erythrocyte radioactivity was corrected for the amount removed by sampling.[7] The erythrocyte survival probability functions and the median survival times were calculated by methods previously reported.[3, 4]

TABLE 1—Data on Experimental Animals and the Amount of Isotope Injected

Experimental animal	Age (mo.)	Sex	Wt. (kg.)	PCV*	Isotope injected (μc./kg.)
White-faced sheep 516**	8	F	45.0	37.5	13.3
White-faced sheep 60**	8	F	33.0	37.5	4.5
Southdown sheep 720	6	F	27.3	34.0	18.3
Karakul sheep	24	F	36.0	38.0	15.4
Bighorn sheep	12	F	31.8	47.0	15.7

* Mean packed cell volume during the course of the experiment. ** Grade Corriedale-Columbia.

Results and Discussion

The survival probability functions $[p(t)]$ for labeled erythrocytes of 3 domestic sheep are presented graphically (Fig. 1). The median survival times $p(t\,^1/_2)$ of the erythrocytes of sheep 720, 60, and 516 are 64 days, 94 days, and 118 days, respectively. Similar graphs for a Karakul sheep (Fig 2) and bighorn sheep (Fig. 3) provide estimates for median survival times of 130 days and 147 days, respectively, in these animals. The results of this investigation indicate that considerable variation may occur in survival times among the erythrocyte populations of the various breeds as well as between the ovine species. Mean erythrocyte survival times for sheep have been estimated to be about 55 days, utilizing the indirect Fe^{59} technique.[1]

Inspection of the graphs (Fig. 1, 2, 3) indicates that a quasi linear rate of decrease of specific activity of hemin occurred in all animals in this investigation. The estimated rates of decrease were comparable to a rate of loss of erythrocytes between 0.75 and 1.50% per day. Thus, it would appear that the variations in survival times more closely reflected the length of time during which the erythrocytes were partially resistant to the processes of destruction. This phenom-

Received for publication Aug. 8, 1960.

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This study was supported in part by the Biological Research Institute of the Zoological Society of San Diego through an Ellen B. Scripps Fellowship.

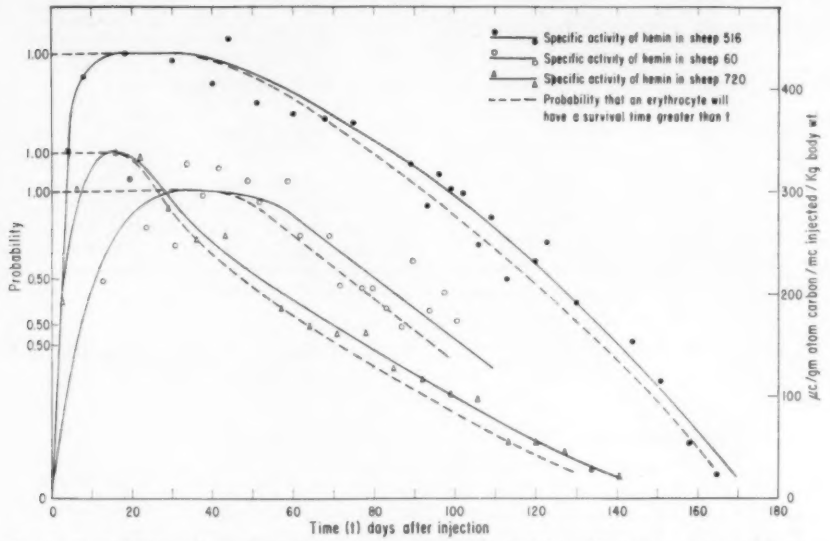
Specific activity of hemin in sheep 516
Specific activity of hemin in sheep 60
Specific activity of hemin in sheep 720
Probability that an erythrocyte will have a survival time greater than t
Probability
μc./gm atom carbon / mc injected / Kg body wt.
Time (t) days after injection

Fig. 1—Probabilities of erythrocyte survival and specific activities of hemin in 3 sheep following injection of glycine-2-C.[14]

enon would be reflected in a curve showing little or no change in specific activity of hemin with respect to time followed by a period of linear loss of radioactivity. The graphs of the results of this investigation show that for all sheep except No. 720 there were varying lengths of time during which the specific activity remained relatively constant. The continuous synthesis of heme from glycine-2-C[14] should not have contributed significant radioactivity to the circulating heme pool during the course of this study. It has been shown that the specific activity of the free glycine pool decreases

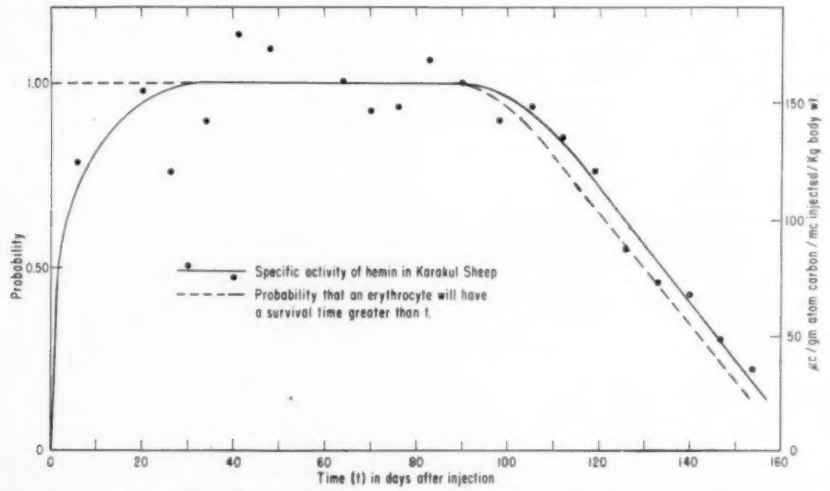
Specific activity of hemin in Karakul Sheep
Probability that an erythrocyte will have a survival time greater than t.
Probability
μc./gm atom carbon / mc injected / Kg body wt.
Time (t) in days after injection

Fig. 2—Probability of erythrocyte survival and specific activity of hemin in a Karakul sheep.

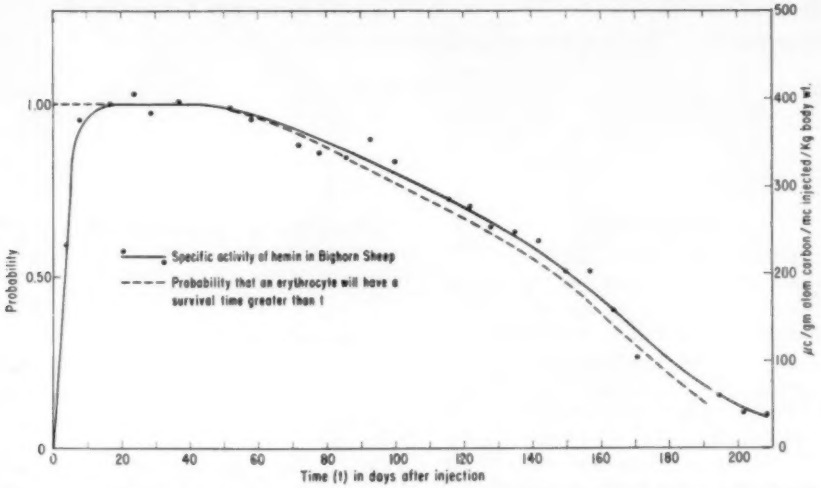

Fig. 3—Probability of erythrocyte survival and specific activity of hemin in a bighorn sheep.

exponentially after the intravenous injection of labeled glycine into cows.[2]

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SUMMARIO IN INTERLINGUA

Studios del Superviventia de Erythrocytos in le Ove Domestic e le Ove del Montanias Roccose, con le Uso de Glycina-2-C^{14}

Le superviventia del erythrocytos esseva studiate in 4 oves domestic e 1 ove del Montanias Roccose. Le tempores median de superviventia esseva 94 e 118 dies in duo agnos a facie blanc de racia cruciate, 64 dies in un agno de racia Southdown, 130 dies in un ove matre de racia Karakul, e 147 dies in un ove del Montanias Roccose.

Serum Transaminase in the Blood of Lambs Given Preventive Treatments for White Muscle Disease

André Lagacé, D.V.M., Ph.D.; D. S. Bell, M.S.; A. L. Moxon, Ph.D.; W. D. Pounden, D.V.M., Ph.D.

SUMMARY

Four groups of lambs, totaling 128, were used to study variations in serum transaminase according to age and white muscle disease preventive treatment.

During the first 4 weeks of life the SGO-T mean of the blood of all lambs stayed between 43 and 53 Sigma-Frankel units. Then, the SGO-T mean of the blood of the lambs whose dams received selenium orally, the lambs given injections of selenium, as well as the controls, rose to a peak of 126 units to come down to 81 units by the 10th week.

The controls and lambs from selenium-fed dams had the highest increase in SGO-T; the lambs given *alpha*-tocopherol had the least.

INTRODUCTION

The enzyme, glutamic oxalacetic transaminase, has been reported to increase in human blood serum following acute myocardial infarction,[3,7,9] necrosis of skeletal muscles,[9,15] and necrosis of the liver.[10] Recently, the determination of this enzyme in blood serum has been found to be a reliable test in the diagnosis of clinical and subclinical white muscle disease (WMD) in lambs.[1,4,8,14] The normal serum glutamic oxalacetic transaminase (SGO-T) range in lambs is less than 200 SGO-T units.[1] Several investigators have reported the preventive value of selenium for WMD when injected or fed to ewes[2,11] during gestation. Selenium was similarly effective when added to the feed or injected into lambs after birth.[2,6] *Alpha*-tocopherol had no preventive value in the ewe ration before parturition[11,12] but was effective when given to lambs after birth.[5]

Received for publication Sept. 15, 1960.

From the Departments of Veterinary Science and Animal Science, Ohio Agricultural Experiment Station, Wooster.

The authors thank Dr. C. R. Weaver for his help in the statistical analysis of the data.

The purpose of this study was to determine the SGO-T levels in the blood of normal lambs and lambs given selenium and *alpha*-tocopherol as WMD preventive treatment.

Materials and Methods

Animals.—The 128 lambs used in this experiment were from a flock usually experiencing a sporadic incidence of WMD. Lambs were allotted to 4 groups: Group 1 consisted of 47 Targhee lambs used as controls; group 2 contained 44 Targhee lambs whose dams had been fed selenium at the rate of 0.1 p.p.m. based on the entire ration; group 3 included 20 Columbia lambs given 1 mg. of sodium selenate subcutaneously every 2 weeks; group 4 consisted of 17 Columbia lambs given 525 mg. of dl-*alpha*-tocopherol-acetate subcutaneously each week. Treatment was continued for 12 weeks. During the experimental period, these animals were confined and fed hay not known to produce WMD.

Drugs.—For parenteral injections, selenium was used as sodium selenate diluted with sterile water so that 2 ml. of the dilution contained 1 mg. of sodium selenate; 1 mg. was given at 14 days of age to all the lambs in group 3 and repeated every 2 weeks until they were 3 months old.

For ewe feeding* (group 2), sodium selenate was dissolved in water and sprayed over 6 lb. of finely ground shelled corn. This was mixed with ground ear corn and fed to provide 0.1 p.p.m. of selenium. Different groups of ewes were fed this ration for varying periods ranging from 2 weeks to 2 months prior to parturition and until 3 months after.

The *alpha*-tocopherol acetate (525 mg.), equivalent to the activity of 525 units of vitamin E, was injected subcutaneously into the lambs in group 4 at 5 days of age and every week thereafter until they were 3 months old.

Biochemistry.—Five milliliters of blood was taken from the jugular vein of each lamb at 1, 7, 14, 28, 42, 56, 70, and 84 days of age. The serum was collected, frozen, and used for SGO-T determinations within 7 days. The technique used for these determinations was essentially the same as described in Technical Bulletin No. 505, Sigma Chemical Company.[13] The only difference was the addition of 15 ml. instead of 10 ml. of 0.4 N sodium hydroxide to develop the color.

* Recommended in personal communication from Dr. O. H. Muth, Oregon State College, Corvallis, in 1960.

TABLE 1—Analysis of Variance of the SGO-T Levels of the Blood Serum at Different Ages in 4 Groups of Lambs

Source of variation	Σ squares	Degrees of freedom	Mean squares	F
Total	29,205			
Treatments	1,223	3	408	2.598 sig. 06
Periods	22,138	7	3,163	20.148**
Periods linear	13,618	1	13,618	86.747**
Periods quadratic	2,858	1	2,858	18.206**
Residue p	5,662	5	1,132	7.211**
Treatment × periods	5,844	21	278	1.771*
T × periods linear	137	3	46	1 n.s.
T × periods quadratic	2,675	3	892	5.682**
Residue tp	3,032	15	202	1.297 n.s.
Error		903	157	

* Indicates significance at the 0.05 level of probability; ** indicates significance at the 0.01 level of probability; n.s. = not significant.

Results

The SGO-T mean of the blood samples taken the first 4 weeks of life stayed between 43 and 53 *Sigma*-Frankel units. Between the 4th and 6th week, the SGO-T mean of groups 1, 2, and 3, climbed to a peak of 126 units, stayed high between the 6th and 8th week of age, and leveled off to 81 units by the 10th week (Fig. 1). Seventy per cent of the lambs showed this increase in their SGO-T values.

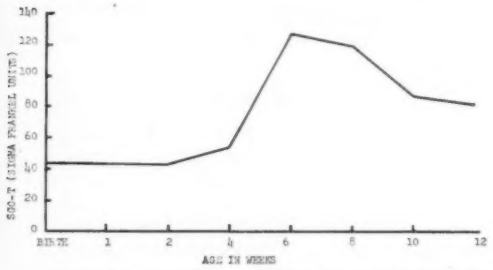

Fig. 1—Variations in the SGO-T mean level of the blood serum according to age (groups 1, 2, 3).

This elevation in the SGO-T levels after the 4th week of life was common to all 4 experimental groups of lambs but the lambs given only *alpha*-tocopherol had a slow and gradual increase to a level of 90 units by the 10th week of life. The controls and lambs from selenium-fed dams were found to have the highest increase while the increase for lambs given selenium injections was intermediate (Fig. 2).

An analysis of variance of the unweighted means used in Figure 2 was done to fortify the data (Table 1). From this analysis, the following items were concluded: (1) The SGO-T levels increase with age (period linear); (2) the SGO-T levels X age relation is

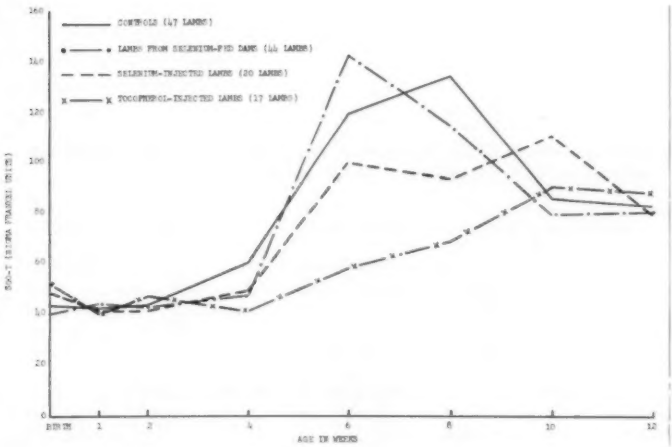

Fig. 2—Variations in the SGO-T levels of the blood serum according to age in 4 groups of lambs.

a curve (period quadratic); (3) there is some unexplained relationship of SGO-T levels to age (residue); (4) the SGO-T levels X age relation increases in each treatment (treatment X period linear interaction); (5) the SGO-T levels X age relation curves differently depending on the treatment (treatment X period quadratic interaction).

Discussion

None of the lambs, treated or untreated, developed signs of WMD. However, in most lambs, an elevation of the SGO-T levels in their blood was noticed between the 4th and 10th week of age, the higher levels being in the 6th or 8th week. This elevation took place in the period when WMD would be expected to occur. This is believed to be manifestations of subclinical WMD since the relatively large doses of *alpha*-tocopherol reduced these subclinical increases.

Selenium fed to ewes in gestation has been shown to prevent WMD in their lambs [6,11]; however, in this experiment, it did not inhibit an increase in the SGO-T readings. However, 1 mg. of selenium injected into lambs every 14 days did reduce the SGO-T levels (Fig. 2). The results indicate that, under the conditions of our flock, injections in lambs of either *alpha*-tocopherol or sodium selenate would be expected to help in the prevention of WMD; the use of sodium selenate would be more economical.

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SUMMARIO IN INTERLINGUA

Nivellos Seral de Transaminase in le Sanguine de Agnos, sub le Influentia de Tractamento Preventive Contra Morbo de Musculo Blanc

Quatro gruppos de agnos—un total de 128—esseva usate pro studiar variationes in le transaminase seral secundo le etate e le absentia o presentia de tractamento preventive contra morbo de musculo blanc.

Durante le prime 4 septimanas del vita, le nivellos de transaminase glutamic-oxaloacetic in le sero remaneva pro omne le agnos inter 43 e 53 unitates sigma-Frankel. Postea, le valor medie de transaminase glutamic-oxaloacetic del sero montava a un maximo de 126 unitates in le sanguine de agnos con matres tractate con selenium per via oral, de agnos tractate directemente con injectiones de selenium, e etiam del agnos de controlo, sed al fin del decime septimana ille valor habeva redescendite a 81 unitates.

Le agnos de controlo e le agnos de matres tractate con selenium oral habeva le plus marcate augmentos de transaminase glutamic-oxaloacetic del sero. In le agnos tractate con *alpha*-tocopherol, ille augmento esseva le minus marcate.

Variation in the Inflammatory Response and Bactericidal Activity of the Sheep Uterus During the Estrous Cycle

H. W. Hawk, Ph.D.; G. D. Turner, B.S.; J. F. Sykes, Ph.D.

SUMMARY

Estrous and luteal-phase sheep in 20 pairs were inoculated *in utero* with *Escherichia coli* and killed 4 to 24 hours later. Uterine defenses were evaluated by the leukocytic response and bactericidal activity. The leukocytic response developed significantly more slowly in luteal-phase sheep and *Esch. coli* were cleared more slowly from the uterine lumens. The differences between estrous and luteal-phase sheep were greatest at 8 hours postinoculation, then decreased in magnitude as the inflammatory response developed in luteal-phase sheep.

INTRODUCTION

The ovarian status has a marked effect on the defensive mechanisms of the rabbit uterus during the early stages of induced infection.[3-7, 10, 13] The leukocytic response is inhibited in luteal-phase rabbits and bactericidal activity in the uterine lumen is delayed.

This report is concerned with the results of an investigation on the influence of the estrous cycle on the inflammatory response and bactericidal activity of the sheep uterus. Preliminary results were reported previously.[9]

Experimental Procedures

The ewes were predominantly crossbreds involving the Shropshire, Hampshire, Southdown, and Merino breeds. Their mean age was 4 years. Most had lambed at least once.

Forty ewes were used in 20 pairs, an estrous and a luteal-phase ewe in each pair. Each estrous ewe stood for mounting on the day of use, but copulation was prevented by the use of aprons on the rams. Luteal-phase ewes were used 7, 8, or 9 days after estrus. The ewes were paired as nearly as possible with regard to age.

Received for publication Oct. 31, 1960.

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The authors are grateful to personnel of the Sheep Research Branch, USDA, Beltsville, Md., for supplying the sheep used in this study.

Each ewe was anesthetized with pentobarbital sodium, and ether was used as needed. A midventral incision was made, and 1 uterine horn was ligated midway between the internal and external bifurcations. Through an incision made in the oviduct near the tubo-uterine junction, a blunt needle was passed into the uterine lumen and an *Escherichia coli* suspension was injected. A ligature was tightened around the tubo-uterine junction as the needle was withdrawn.

The 2 ewes of each pair were inoculated within a few minutes of one another with the *Esch. coli* suspension.

Bacterial suspensions were prepared by washing a 24-hour culture of *Esch. coli* from a trypticase soy agar slant with sterile 0.87% NaCl solution and diluting to a standard turbidity.

The inoculum per uterine horn was usually about 500 million *Esch. coli* cells in 0.5 ml. of saline solution. Inoculums of over 1 billion cells per ewe were used for 3 pairs of ewes.

Six pairs of ewes were killed 4 hours after uterine inoculation; 8 pairs at 8 hours; 1 pair at 12 hours; 2 at 16 hours; and 3 at 24 hours. Data from the 6 pairs killed between 12 and 24 hours were grouped for statistical analysis.

Immediately after slaughter, sterile saline solution was injected through a seared area on the serosa of each inoculated horn. Ten milliliters of solution were injected into each horn of luteal-phase ewes. Usually, lesser volumes were injected into horns of estrous ewes because of accumulated fluid secretions. Each horn was manipulated vigorously to dislodge *Esch. coli* cells and leukocytes from among caruncles and to suspend them in solution; it was then drained of its contents and the volume was recorded.

The uterine flushings were diluted serially and 1-ml. aliquots of each dilution were plated in violet red bile agar. The plates were incubated at 37 C. for 18 to 24 hours, and the colony counts were used to calculate the total number of live *Esch. coli* flushed from each uterine horn.

Aseptic techniques were used throughout the inoculation and flushing of the uterine horns and enumeration of the *Esch. coli*.

Numbers of leukocytes in the flushings were estimated by microscopic counts or by packed cell volume.[8]

Specimens from both the inoculated and uninoculated uterine horns of each ewe were fixed in Bouin's solution. Sections were cut at 9 μ and stained with hematoxylin and eosin. The relative intensity of leukocytic infiltration of each uterine horn was graded microscopically on a scale from

0 to 8. A lack of infiltration was 0, slight infiltration was 1, etc., with a grade of 8 indicating an extremely dense concentration of leukocytes in the endometrium. Several tissue sections were examined from each uterine horn, but little variation in infiltration was noticed among sections from the same horn.

Results

The mean degrees of leukocytic infiltration of the endometrium and mean numbers of leukocytes and live *Esch. coli* in the uterine flushings are listed (Table 1).

TABLE 1—Numbers of Escherichia coli and Leukocytes Flushed from Sheep Uteri after Experimental Inoculation, and Relative Degrees of Endometrial Leukocytosis

Hours after inoculation	No. of pairs	Endocrine state: Estrous — Tissue leukocytosis	Estrous — Luminal leukocytes	Estrous — *Esch. coli*	Luteal — Tissue leukocytosis	Luteal — Luminal leukocytes	Luteal — *Esch. coli*
4	6	3.3*	27.5**	245.0**	1.5	7.9	316.0
8	8	6.4	115.0	19.5	3.6	25.1	282.0
12–24	6	5.7	251.0	0.3	5.5	245.0	3.7

* Values indicate relative degrees of leukocytic infiltration graded on a scale from 0 to 8; 0 indicates no detectable response and 8 indicates that polymorphonuclear leukocytes packed the subepithelial endometrium.
** Values indicate millions of leukocytes or *Esch. coli* corresponding to the mean log of individual observations.

At 4 hours postinoculation, in estrous ewes there was moderate infiltration of the endometrium by polymorphonuclear leukocytes. Infiltration in luteal-phase ewes was relatively slight. The difference was significant statistically ($P<0.05$) as analyzed by within-pair differences in indexes of leukocytic infiltration.

The numbers of leukocytes in the uterine lumens were also significantly greater in estrous ewes ($P<0.01$), as shown by within-pair differences in the log numbers of leukocytes in the uterine flushings. However, the leukocytic influx was not sufficiently advanced for marked bactericidal activity to be seen in estrous ewes. The numbers of live *Esch. coli* were still large in each type of ewe. Some phagocytized *Esch. coli* were seen within leukocytes in the uterine flushings.

At 8 hours postinoculation, marked differences were found between estrous and luteal-phase ewes, both in intensity of the leukocytic response and in uterine bactericidal activity. Tissue leukocytosis was again greater in the estrous ewes than in luteal-phase ewes ($P<0.01$). More important, the number of leukocytes in the uterine lumen was much greater in the estrous ewes, the mean log number being 8.06 (115,000,000) compared to 7.40 (25,100,000) for luteal-phase ewes ($P<0.01$). Almost all of these leukocytes were polymorphonuclear.

The mean log number of *Esch. coli* flushed from uteri of estrous ewes, 7.29 (19,500,000), was significantly less ($P<0.05$) than the mean for luteal-phase ewes, 8.45 (282,000,000). Of the 8 pairs of sheep killed at this stage, the uteri of 7 of the estrous ewes contained the lesser numbers of *Esch. coli*.

Phagocytosis of *Esch. coli* by polymorphonuclear leukocytes was evident in all of the sheep killed at 8 hours. Little difference was noticed between estrous and luteal-phase ewes in the number of phagocytized *Esch. coli* per unit number of leukocytes. The total number of phagocytized *Esch. coli* was, therefore, much greater in the estrous ewes.

By 12 to 24 hours, the leukocytic response and uterine bactericidal activity were well advanced in luteal-phase ewes as well as in estrous ewes. Leukocytic infiltration of the endometria and numbers of leukocytes in the uterine lumens were nearly equal in each type of ewe (Table 1). The numbers of *Esch. coli* in the uterine flushings were relatively small; the mean log number for estrous ewes was 5.44 (275,000), and for luteal-phase ewes was 6.57 (3,720,000). This difference was significant statistically ($P<0.05$) but did not approach in magnitude the difference at 8 hours.

Phagocytized *Esch. coli* were seen in some leukocytes of each kind of ewe killed at 12 and 16 hours. At 24 hours, few *Esch. coli* cells could be found in leukocytes. Digestion of *Esch. coli* phagocytized earlier had presumably progressed beyond the point that such cells could be recognized in leukocytes.

Discussion

Sheep are similar to rabbits with regard to the influence of the ovarian status on the rapidity of the inflammatory response to induced infection in the uterus. Also, the leukocyte-bacteria numerical relationships in sheep after 12 to 24 hours were similar to those found previously in cows studied 24 to 48 hours after experimental uterine inoculation.[1,12]

The present results may be somewhat at variance with those of Lamming and Heap,[11] who reported that disappearance of bacteria from lumens of ligated uteri was similar in estrous and luteal-phase sheep.

A longer time was required in sheep than in rabbits for the development of a highly effective uterine defense against *Esch. coli*.[3,5,7,10] In general, a smaller number of bacteria per unit of body weight was used in sheep than in rabbits. However, larger inoculums used in 1 pair of sheep killed at 4 hours and 2 pairs killed at 8 hours did not evoke an appreciably greater leukocytic response. The species difference was probably due to factors other than variation in numbers of bacteria relative to body size.

Phagocytosis by polymorphonuclear leukocytes undoubtedly constituted a major uterine defense against the *Esch. coli* in uterine lumens of each kind of sheep. Since the amount of phagocytosis was roughly proportional to the numbers of leukocytes in the uterine lumen, the differential rates of leukocytic influx in estrous and luteal-phase ewes accounted at least in part for the faster clearance of *Esch. coli* from estrous ewes. Several investigations have shown the more efficient uterine defenses of estrous as compared to pseudopregnant rabbits to be associated with an earlier leukocytic response.[3,5,7,10]

Somewhat greater numbers of *Esch. coli* were alive in luteal-phase sheep at 12 to 24 hours, despite leukocyte numbers as great as in estrous sheep. This was probably due to bacterial multiplication, but could also have been due partly to less effective bactericidal activity by leukocytes in luteal-phase sheep or to greater bactericidal activity by nonleukocytic factors in estrous sheep.

A statistical evaluation of the relationship between leukocytic influx and uterine bactericidal activity was obtained by using log numbers of *Esch. coli* and leukocytes flushed from the uterus. The correlation coefficient was significant over all sheep ($r = -0.499$, $P < 0.01$). However, the relationship was higher in luteal-phase sheep ($r = -0.747$, $P < 0.01$) than in estrous ones ($r = -0.229$, $P > 0.05$), and the difference between these correlation coefficents was significant ($P < 0.05$). These relationships are similar to those obtained by Winter *et al.*[13] in follicular-phase and pseudopregnant rabbits.

The biological significance of the lower correlation coefficient in estrous sheep may be doubtful in view of the clearance of *Esch. coli* from uteri of estrous ewes between 4 and 8 hours coincident with the influx of large numbers of leukocytes. Nevertheless, the possibility should not be discounted that extra-leukocytic defensive mechanisms might have been active in ligated uteri of the estrous sheep.

In a limited number of tests made in the present study, cell-free ovine uterine exudates were bactericidal *in vitro*. The role of the noncellular bactericidal factors is not certain. They have been found previously in inflammatory exudates of rabbit uteri and seem to have some defensive value.[5,8,13] The bactericidal potency of cell-free rabbit exudates was related to the degree of leukocytic influx.

Why the leukocytic response developed more slowly in luteal-phase than in estrous sheep is not known. It was probably not due to differences in the normal infiltration of the endometrium by polymorphonuclear leukocytes. In uninoculated uterine horns of each kind of sheep, there was little infiltration by this type of leukocyte. In rabbits, the differential leukocytic response in estrous and luteal-phase animals could not be accounted for by differences in numbers of leukocytes normally present in the uterus,[4] by differences in numbers of circulating leukocytes,[4] or by a lack of leukotaxic substances in inoculated uterine lumens of pseudopregnant rabbits.[10]

The slower influx of leukocytes into inoculated uterine lumens of pseudopregnant than of estrous rabbits was due to slower passages of leukocytes through the endometrial tissue as well as to a less intense initial response.[4,7,10] In the present study, the early leukocytic response was much less intense in luteal-phase ewes, but it was not possible to determine with any degree of certainty whether leukocytes passed

through endometriums of the 2 kinds of sheep at different rates.

Differences between estrous and luteal-phase rabbits and cows in response to experimental uterine infections are due more to inhibition of the uterine defenses by progesterone than to stimulation by estrogens.[2, 7, 10, 12] This is probably also true in sheep, but further study is needed to elucidate the roles of estrogen and progestin in controlling the uterine defenses in this species.

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SUMMARIO IN INTERLINGUA

Variation, Durante le Cyclo Estral, in le Responsa Inflammatori e le Activitate Bactericida del Utero Ovin

Oves estral e de phase luteal in 20 pares esseva inoculate in utero con *Escherichia coli* e sacrificate inter 4 e 24 horas plus tarde. Le defensas uterin esseva evalutate per le responsa leucocytic e le activitate bactericida. Le responsa leucocytic se disveloppava plus lentemente in oves de phase luteal, e *Esch. coli* esseva eliminate plus lentemente ab le lumine uterin de illos. Ambe iste differentias esseva statisticamente significative. Le differentias inter oves estral e oves de phase luteal esseva le plus grande 8 horas post le inoculation. Postea illos declinava in magnitude durante que le responsa inflammatori se disveloppava in oves de phase luteal.

Bovine Achondroplasia. III. Standards of Metacarpal Indexes for Achondroplastic, Brachycephalic Dwarfs and Controls

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SUMMARY

Standards of 3 metacarpal indexes for brachycephalic dwarf and control Hereford cattle are presented. The indexes are objective criteria of bovine achondroplasia which may be used diagnostically to identify the phenotype of animals resulting from progeny tests or of animals used for investigations of dwarfism. Less than 3% of the values for dwarfs overlap the values for control stocks. Correction factors for age and sex are unnecessary, as the indexes are relatively independent of the influence of these factors. The basic osteologic lesion of achondroplasia, a short diaphyseal length combined with normal epiphyseal length, is responsible for the differences between the metacarpal indexes of brachycephalic dwarf and control cattle.

INTRODUCTION

This report presents standards of 3 metacarpal indexes which are objective criteria of bovine dwarfism as they apply to achondroplastic, brachycephalic dwarf and control cattle. The brachycephalic dwarf was first described by Johnson *et al.*[17] Gregory *et al.*[12] later presented a more complete description. This type of bovine dwarf has also been referred to as the "snorter," or short-headed, dwarf. The dwarfing process was identified as achondroplasia by Julian *et al.*[18] and confirmed by Tyler *et al.*[26] Biochemical data recently obtained supports this diagnosis.* The relationship of the brachycephalic dwarf to other dwarf forms has been discussed.[8,9,14,19,21] Several theories of the inheritance and suggested methods of control have been presented.[11,13,15,22,23] The uncertainties of physiologic[7,10] and of anatomical[2,11] criteria for the differentiation of "carrier" and "clean" cattle has emphasized the importance of carefully controlled progeny tests.[15] Objective criteria for the phenotypic identification of all progeny resulting from such tests are essential to their uniform interpretation.

Materials and Methods

Metacarpal bones of 318 cattle were measured for this study. The 139 brachycephalic dwarfs were allotted to 2 groups, according to the presence or absence of fusion of the distal metacarpal synchondrosis. Dwarf animals were identified phenotypically by one or more of the following criteria: premature fusion of the spheno-occipital synchondroses,[16] head length/head width ratio of approximately 1:1.75,[19] projection of the posterior intraoccipital synchondrosis or of the orbital wing of the sphenoid,[27] and general appearance. All of the dwarf animals were purebred or grade Herefords.

The 179 control specimens were collected at a local abattoir and had diverse origins. Animals without fusion of the distal metacarpal synchondroses were classified according to sex and age as steers, heifers, or "veal" calves. The control cattle with fused metacarpal synchondroses were all cows. All control animals appeared to be Herefords, except 20 of the veal calves which appeared to be crosses of the beef breeds. The genetic background of the control cattle was unknown.

Positive identification of the metacarpal bones was maintained by the use of plastic tags inscribed with the identification and wired to the diaphysis and epiphyses. The bones were prepared for measurement by maceration in a 1 to 2% solution of sodium hydroxide. All measurements were made with an outside caliper and recorded to the nearest millimeter. The total length (TL) was measured from the proximal articular surface along the vas-

Received for publication Sept. 23, 1960.

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This work was supported in part by USPHS research grant A2626 M&N.

The authors thank members of the University of California Agricultural Extension Service and School of Veterinary Medicine who aided in the collection of specimens. Other specimens were obtained from the University of California dwarf herd which has been maintained in part by the Kellogg Fund of the University of California for the Application of Genetics to Farm Animals. The technical assistance of Mr. Peter Manelis and Mr. Richard Robenalt is gratefully acknowledged.

* Unreported data obtained during 1960 by Dr. Karl Meyer, Dr. Walter S. Tyler, and Dr. Paul W. Gregory, University of California, Davis.

cular groove to the most distal medial portion of the medial condyle. The smallest transverse diameter was used as the diaphyseal diameter (DD). Only these 2 measurements were possible if the distal metacarpal synchondroses had fused. If the epiphyses had not fused with the diaphysis, the diaphyseal length (DL) was measured from the same point on the proximal articular surface used in the total length measurement to the most distal portion of the dorsal medial projection located on the distal end of the diaphysis. The points from which the measurements were made are illustrated (Fig. 1). The total length and diaphyseal diameter measurements were made in a manner identical to that used in a previous report.[25] The distal point of the diaphyseal length measurement was changed to a point which resulted in less variation.

Three indexes were calculated from the measurements made on metacarpals with unfused distal synchondroses. Only one of these indexes could be calculated for metacarpals in which the epiphyses had fused to the diaphysis. The TL/DD indexes could be calculated for all of the animals. This index is comparable to the metacarpal length/smallest width index of the previous study.[25] The other indexes, which involve the diaphyseal length, are not comparable in the 2 studies since the distal measuring point was changed. The relationship of the total and the diaphyseal length was revealed by the TL/DL index. One new index, the DL/DD, has been introduced.

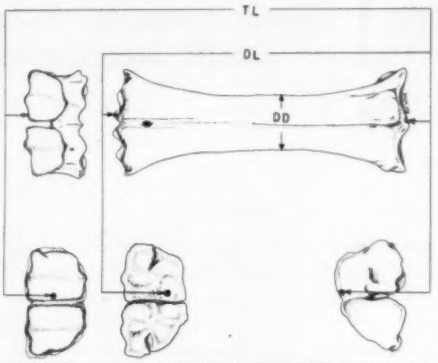

Fig. 1—Bovine metacarpal measurements. TL = total length; DL = diaphyseal length; and DD = diaphyseal diameter.

Results

The results are presented graphically (Fig. 2). Only 4 brachycephalic dwarfs (2.8%) had metacarpal indexes within the range of values of the control stocks. The overlap of dwarf values occurred 3 times in the TL/DL index, 4 times in the TL/DD index, and twice in the DL/DD index.

Analysis of variance revealed significant differences among the means of the samples. Therefore, the means of all possible pairs of samples were tested independently, using appropriate large or small sample methods.[1,24] A statistical summary is presented (Table 1). Significant differences were not found between the Hereford and crossbred veal calves, with the exception of the TL/DL index. Significant differences of the means were found in all other pairs of samples.

The statistical analysis emphasizes minor differences that are present among the control stocks due to the small dispersion of values and the relatively large sample size. The largest difference occurred between the veal calves and cows in the TL/DD index (Fig. 2). Similar comparisons could not be made in the other indexes, since the cows had fused distal metacarpal synchondroses and values for these indexes could not be obtained. The observed differences may be

TABLE 1—Summary of Statistical Significance of the Difference of the Means

Comparison	d.f.	TL/DL	TL/DD	DL/DD
Control Hereford veal calves vs. control non-Hereford veal calves	43	t = 2.65*	t = 0.38	t = 0.81
Brachycephalic dwarfs vs. control veal calves, combined	....	24.14**	26.40**	28.30**
Brachycephalic dwarfs vs. control heifers	....	33.15**	27.12**	29.97**
Brachycephalic dwarfs vs. control steers	....	36.40**	26.71**	31.72**
Control veal calves, combined vs. control heifers	....	5.71**	6.08**	5.19**
Control veal calves, combined vs. control steers	....	8.04**	12.09**	10.93**
Control heifers vs. control steers	....	2.76**	7.25**	6.72**
Control heifers vs. control cows	....	.....	5.22**	.....
Control cows, fused, vs. brachycephalic dwarfs, fused	60	.....	t = 16.33**	.....

Numbers represent values of z or Student's "t" test; z values >1.96 indicate significance at 5%, and z values >2.58 indicate significance at 1%. d.f. = Degrees of freedom.

* significant at 5%.

** significant at 1%.

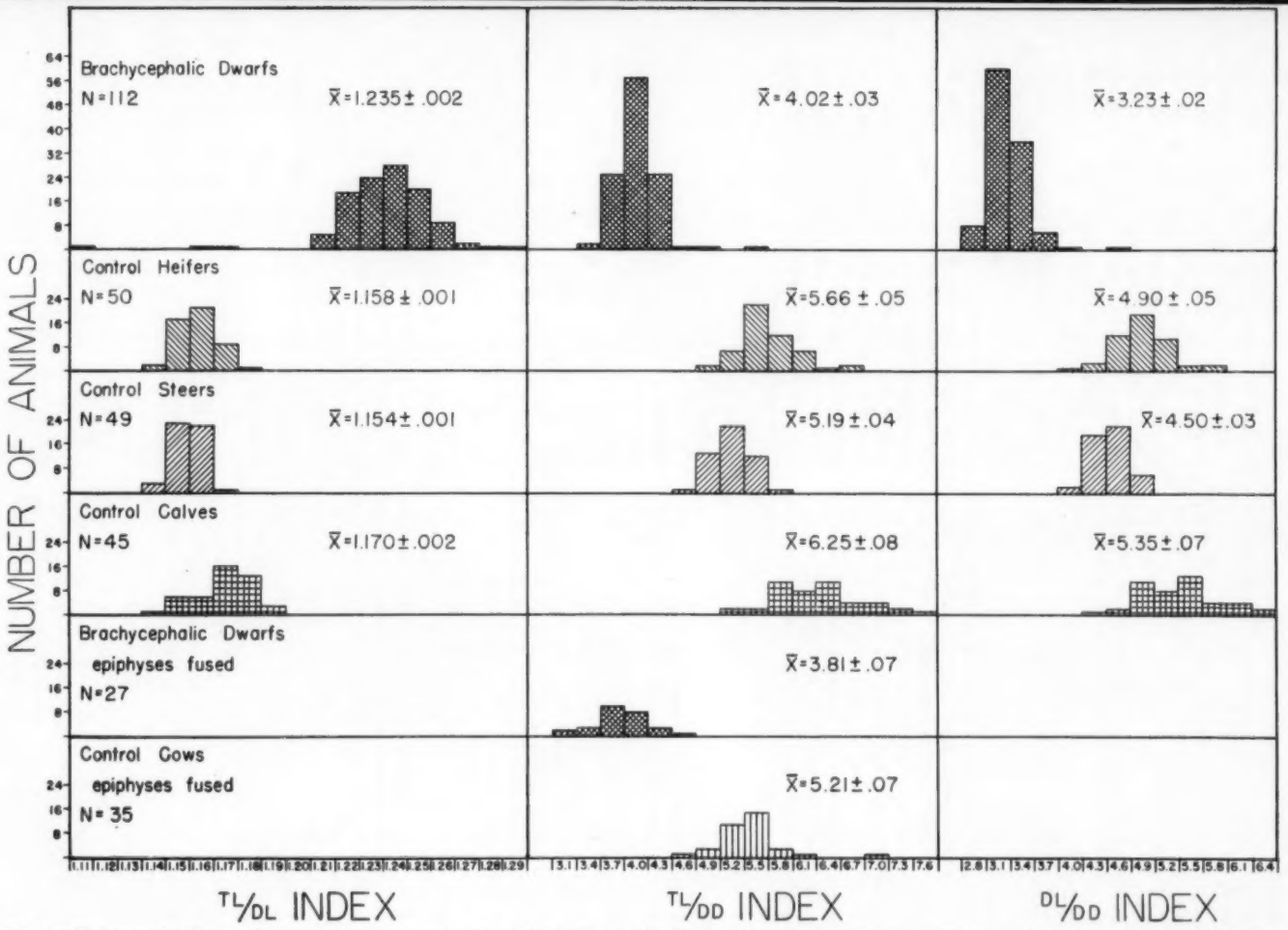

Fig. 2—Metacarpal indexes of brachycephalic dwarfs and control cattle. TL/DL = total length/diaphyseal length; TL/DD = total length/diaphyseal diameter; and DL/DD = diaphyseal length/diaphyseal diameter.

due to differences in age or sex, or they may be due to the selection of animals for early or late slaughter.

Suggested standards for these indexes are presented (Table 2). In establishing these standards, the total range of the control sample was used and the small variation among the various control stocks was not considered, as it could have influenced the decision on only 2 of the 4 brachycephalic dwarfs whose metacarpal indexes overlapped those of the control animals.

TABLE 2—Standards of Metacarpal Indexes for Brachycephalic Dwarf and Control Cattle

	TL/DL	TL/DD	DL/DD
Brachycephalic dwarfs	1.21–1.29	3.3–4.4	2.7–3.8
Control cattle	1.14–1.19	4.7–7.5	4.1–6.4

Analysis of variance among the brachycephalic dwarfs did not reveal significant differences due to age or sex in any of the 3 indexes.

Discussion

Differences in the metacarpal bones of dwarf and normal cattle have been previously reported and used for the diagnosis of bovine dwarfism.[16,23,26] Standards were not established, as only small numbers of animals were measured. In some studies, the measurements were performed on living animals and probably involved several anatomical units.[2,16,23] Generally, measurements on living animals are more subject to error and to interpretation by the observer due to overlying soft tissues than are measurements on prepared bones. The authors have been unable to find reports of measurements of diaphyseal lengths in living cattle.

The significance of independent measurements of the diaphysis and epiphyses has been discussed by Crew [5,6] and others.[20,25,26] The micromelia of this type of bovine dwarfism and of the Dexter dwarf is due to the small size of the diaphyses or primary centers of ossification; the epiphyses or secondary centers of ossification are relatively normal.[5,6,26] These relative changes in the contribution of the diaphysis and epiphyses to the total length are detectable in all appendicular skeletal bones. The metacarpal bones are used diagnostically, as they have the most pronounced changes [26] and specimens from control stocks are relatively easy to obtain since they are discarded at slaughter.

The use of indexes, rather than absolute measurements, is most important since they reveal specific alterations of normal growth.[26] Age, breed, and sex have only minor influences on these indexes and do not require corrective factors or interfere with their diagnostic use. The 3 indexes should be applied to all specimens with unfused distal metacarpal synchondroses, as each indicates qualities of the metacarpal bone which are influenced by different growth processes. The TL/DL index indicates the relative contribution of the diaphysis to the total length and is especially important in achondroplasia. The TL/DD and DL/DD indexes reveal influences of the dwarfing process on both bone length and body weight, as bone diameters have been demonstrated to be related to body weight.[26] While these 2 indexes are obviously somewhat similar, both are useful. The TL/DD index is the only one that may be applied to animals of all ages, since the distal metacarpal synchondroses fuse at approximately 2½ years of age, and diaphyseal length measurements cannot be made in older animals. The DL/DD index differs, as it is not influenced by the relatively normal epiphyseal length of the dwarf; this index may be used if only the diaphysis is available for study.

These 3 indexes of the metacarpal bones may be considered diagnostic of brachycephalic bovine dwarfism, as less than 3% of the values for 139 dwarfs were within the range of values for 179 control animals. This small overlap may be due to variation in the expression of the dwarfing process similar to that reported by other workers.[3,4] These animals had the general appearance, but only some of the other criteria, that identify brachycephalic dwarfs.

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SUMMARIO IN INTERLINGUA

Achondroplasia Bovin. III. Standards del Indices Metacarpal pro Achondroplastic Nanos Brachycephalic e pro Animales de Controlo

Es presentate standards de 3 indices metacarpal pro nanos brachycephalic e animales de controlo in bestial Hereford. Le indices es criterios objective de achondroplasia bovin. Como tales illos pote esser usate diagnosticamente pro identificar le phenotypo de animales resultante de tests de progenie o de animales usate in le investigation de nanismo. Minus que 3% del valores pro nanos cade in le campo de valores pro bestial de controlo. Factores de correction pro etate e sexo non es necessari proque le indices es relativemente independente del influentia de iste factores. Le basic lesion osteologic de achondroplasia, i.e. un curte longor del diaphyse in combination con un normal longor del epiphyse, es responsabile pro le differentias inter le indices metacarpal de nanos brachycephalic e bestial de controlo.

Nocardia asteroides as a Mammary Pathogen of Cattle. II. The Sources of Nocardial Infection and Experimental Reproduction of the Disease

A. C. Pier, D.V.M., Ph.D.; E. H. Willers, D.V.M.; M. J. Mejia, B.S.

SUMMARY

Nocardia asteroides was isolated from corral soil, infusion cannulas, medicine vials, and partially used drug mixtures, designed for intramammary infusion, on premises where cattle contracted nocardial mastitis. The frequent occurrence of the natural disease in multiparous cattle shortly after parturition linked infection with the introduction of the organism at the time of drug infusion into the nonlactating mammary gland.

Nocardia asteroides was added to mixtures of drugs frequently used for intramammary infusion. The organism was shown to be viable in these mixtures for 7 weeks. Infusion of these experimentally contaminated drug mixtures into a nonlactating mammary gland produced a clinical response typical of the naturally occurring infection.

The institution of proper aseptic techniques and the substitution of disposable, single-dose applicators for multiple-dose drug mixtures alleviated the infection rate in involved herds.

INTRODUCTION

Nocardia asteroides has been recognized as the etiologic agent of a severe bovine mammary infection in a number of California and Hawaiian dairy herds. Reports of the infection in 7 of these herds have been published.[6,7] In 3 of the reported herds, the infection was seen to involve an appreciable number of the animals.

In all herds infected by *N. asteroides*, frequent isolations were made of such common bacterial pathogens as staphylococci, streptococci, pseudomonads, and coliform bacilli. *Nocardia asteroides* was isolated from glands shedding one or more of these pathogens and from glands in which no other pathogen was recognized.

The habitat of *N. asteroides*, when not in animal tissue, is the soil.[3,4] Like other potential pathogens of similar distribution (e.g., *Pseudomonas aeruginosa*), its occasional presence in the bovine mammary gland might be expected. Yet this gland, susceptible to the invasion of a wide variety of organisms, in only 2 other reported instances[1,5] had been invaded by *N. asteroides* at the time this study began. The following epizootiologic investigation was conducted to determine the mechanisms fostering the establishment of the infection which reached epizootic proportions in 3 herds.

Materials and Methods

The infected herds used in this study have been described.[7] The infecting flora of the mammary glands of the cows in each herd was determined by cultural methods previously described.[6] The clinical history and the stage and number of lactations were recorded for known infected animals. The milking practices, barn sanitation, drug infusion procedures, and other pertinent husbandry procedures were examined in each herd. In herds A and F, where infection was extensive, the following materials were collected and examined culturally for *N. asteroides:* the soil of holding corrals; flies captured on cows and in the milking barn; dust from barn rafters, dust from the flanks and udders of cows, samples of feed concentrate, water from corral troughs and milking barn outlets, swabs from the mouths of suckling calves and milkers' hands, teat infusion cannulas, medicine vials, and partially used quantities of drugs prepared for infusion.

Soil, flies, feed, and other particulate samples were suspended in sterile saline solution and shaken thoroughly. The supernatant suspensions were cultured for *Nocardia*.

Over-growth by faster growing organisms from these contaminated sources proved a difficult problem when searching for the slower-growing *Nocardia*. The following 5 methods of cultural isolation were used:

Received for publication Nov. 7, 1960.

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This work was supported in part by research grant No. E-2306 from the National Institutes of Health.

1) Direct streaking was made on tryptose agar containing 5% washed bovine erythrocytes. Ninety-six hours of incubation at 37 C. were allowed before examination.

2) Direct streaking was made on Saboraud's dextrose agar. Ten to 14 days of incubation at 37 C. were allowed prior to examination.

3) Inoculations of suspensions made in sterile saline solution were made into guinea pigs. These animals were killed in 14 days and material from lesions was streaked on tryptose blood agar.

4) Sohngen's carbon-free liquid medium, prepared as described by Frey and Hagan,[4] was inoculated with saline suspensions of the material under examination, and a glass rod coated with sterile paraffin was added to the mixture. These preparations were incubated for 14 to 21 days. *Nocardia* appeared as tuft-like growths on the coated rods from which they were picked and isolated on tryptose blood agar.

5) A plating medium, devised after considerable experimentation with various inhibitory substances, allowed direct isolation of *Nocardia* from saline suspensions of soil samples streaked over the surface. The medium was tryptose agar containing sodium 1,3-dichloro-2-hydroxy-2-propanesulfonate at a final concentration of 28 mg. percent and crystalline penicillin G to a final concentration of 0.5 unit per milliliter. All of the herd strains of *N. asteroides* grew well on this medium when incubated at 37 C. for 14 days. The medium prevented the growth of *Staphylococcus aureus* and *Ps. aeruginosa*, both of which interfered with nocardial isolations. Additional growth selection with this medium was possible when the incubation temperature was 46 C.

Results

In herd A, where the infection was first diagnosed, 2 mammary quarters shed *Ps. aeruginosa*, while the glands of 28 cows shed *N. asteroides* from 1 or more quarters. Similarly, in herd F, *Ps. aeruginosa* was cultured from the milk of 10 cows, *Geotrichum sp.* and *Candida albicans* from 1 cow each, and *N. asteroides* from 39 of 152 cows examined during a 1-month study of the herd. The remaining isolates from the animals in both herds were such common pathogens of the bovine mammary gland as *S. aureus* and *Streptococcus agalactiae*, which are less closely identified with soil flora. Thus, by association it was considered that soil contamination of the glands was the probable source of infection. The lobular distribution of lesions in naturally infected glands[6,7] suggested an ascending infection.

The lactation history of infected cows indicated a propensity for the infection to become clinically apparent shortly after parturition and was most frequently seen in animals previously treated by intramammary infusion.

Naturally infected cows, with but 2 known exceptions, were beyond their 1st lactation. The majority were 4th and 5th lactation animals. Repeated isolations were made from two 1st-lactation heifers with typical clinical signs; 1 of these had clinical signs of infection during the 1st week of lactation. The lactation histories for 23 cattle with culturally diagnosed cases in herds A and F are given (Table 1).

Milking procedure included prewashing of the udders with water or disinfectant

TABLE 1—Lactation History of Cattle Naturally Infected with Nocardia asteroides

Cow No.	Herd	Lactation No.	Stage of lactation when clinically affected	Previous treatment by infusion
782	F	2	2 days postpartum	Treated between lactations*
849	F	2	Nonlactating period (4th month)	Treated between lactations
167	F	3	2 days postpartum	Treated between lactations
175	F	3	Nonlactating period	Treated between lactations
408	F	3	Nonlactating period	Treated between lactations
416	F	3	3 days postpartum	Treated between lactations
424	F	3	1 day postpartum	Treated between lactations
475	F	3	2 days postpartum	Treated between lactations
3791	F	4	Nonlactating period	Treated between lactations
3825	F	4	8 days postpartum	Treated between lactations
3916	F	4	1 day postpartum	Treated between lactations
3477	F	5	4 days postpartum	Treated between lactations
3637	F	5	4 days postpartum	None
3791	F	5	Nonlactating period (2nd month)	Treated between lactations
3553	F	6	Nonlactating period	Treated between lactations
3571	F	6	2 days postpartum	Treated between lactations
1885	F	9	2 days postpartum	Treated between lactations
59	A	1	2 weeks postpartum	None
126	A	2	10 days postpartum	Undetermined†
15	A	4	4 months postpartum	Undetermined
24	A	5	1 month postpartum	Undetermined
165	A	6	2 months postpartum	Undetermined
161	A	7	1 day postpartum	Undetermined

* 61.5% of nonlactating cows were being infused. † Promiscuous treatment of nonlactating and lactating animals.

solution in all herds. In herds A and B, the milking machine teat cups were immersed in freshly prepared chlorine solution when transferred from one cow to another.

In herds A, F, and G, where infection reached epizootic proportions, a hospital barn program was maintained wherein recently parturient cows, sick and mastitic cows, and cows undergoing treatment in the nonlactating period were all handled by the same personnel in the same area. Sanitary precautions were either disregarded or were inadequate as the attendants progressed from one animal to the next.

The aseptic practices used with intramammary infusion therapy were found inadequate in herds A, D, E, F, and G. In herd A, the practice was to cleanse the teat end with 70% alcohol prior to infusion. No disinfection of the teat end was used prior to infusion in herds D, E, F, and G.

A single cannula was used repeatedly on as many as 40 cows daily in herd A, with only momentary immersion in 70% alcohol between quarters. This alcohol was changed every 24 to 48 hours and, although it appeared turbid, *Nocardia* were not isolated when it was culturally examined in the laboratory 48 hours later. These observations are indicated (Table 2).

Soil samples taken from the premises of herds A, D, E, and F were culturally positive for *N. asteroides*. The strains isolated from soil were indistinguishable from strain isolated from infected udders on these premises. *Nocardia asteroides* has also been isolated from the soil adjacent to the dairy barn on the Davis campus of the University of California, but no case of nocardial infection has yet been observed in this herd, which is under close veterinary supervision.

Nocardia asteroides was not isolated from the water samples, the mouths of suckling calves, milkers' hands, milking machines, flies, or feed which were examined on the various premises involved. The isolations of *N. asteroides* from the environment of infected herds are listed (Table 3).

In herds where infection was found to be widespread, therapeutic measures for a variety of mammary infections were used extensively in both lactating and nonlactating cows. When adequate sanitary precautions were installed and the use of multiple-dose infusion vials was abandoned, the spread of nocardial infection was promptly curtailed.

In herd A, the use of infusions was too promiscuous to permit detailed evaluation. The infusion of as many as 40 quarters in a single day from a multiple-dose container was common. Culling of infected cows, the introduction of adequate sanitation of teats after milking and prior to infusion, and the use of single-dose infusion units stopped the incidence of new infections approximately 4 months after the initiation of this regimen.

In herd F, a similar situation existed. Extreme use of therapy was employed to control mastitis of staphylococcal and streptococcal origin. Sixty-one percent of the cows reaching the nonlactating period had clinical mastitis which required treatment.

TABLE 2—Summary of Management Practices Where Herds Were Infected with Nocardia asteroides

Herd	Herd size	Estimated total cases	Prewashing of udders before milking	Teat cups sanitized	Adequate asepsis during infusion	Commingled hospital barn
A	157	56	+	+	−	+
B	48	2	+	+	+	−
D	46	12	+	−	−	−
E	70	10	+	−	−	−
F	700	300	+	−	−	+
G	500	180	+	−	−	+

In herd F, *N. asteroides* was isolated from the infusion cannulas and from the cleansing solution in which medicine vials were stored. These vials were used to contain a therapeutic formula prepared by the herdsman for intramammary infusion. In herd D, a pure culture of *N. asteroides* was obtained from a partially used bottle of infusion mixture containing nitrofurazone and penicillin. This bottle contained visible sediment and animal hair, indicating gross contamination through improper treatment procedures.

TABLE 3—Isolations of Nocardia asteroides from Environment of Infected Dairy Herds

Herd	Corral soil	Water supply	Feed	Flies	Infusion utensils
A	+	−	−	−	−
D	+	=	...	...	+
E	+	−	...	...	−
F	+	−	−	−	+

At the peak of the infection, which occurred several months after moving 700 milking cows to newly prepared premises, 39 culturally diagnosed cases of nocardial mastitis were seen in a single month. Control measures consisted of abolition of mutiple-dose infusion preparations and the substitution of disposable, single-dose applicators. Sanitation procedures were instituted in the milking barn, including prewashing of udders, immersion of the milker's hands in chlorhexidine solution and rinsing of teats in a similar solution after milking. The cows pointed to contamination at the time of treatment in the nonlactating period as the mode of infection.

Experimental reproduction of "treatment contamination" of the udder was undertaken by infusing glands during the nonlactating period with strains of *N. asteroides* suspended in drug mixtures containing 100,000 units of penicillin, 200 mg. of dihydrostreptomycin and, in some instances, 15.0 ml. of 0.2% nitrofurazone solution. These mixtures were stored at room temperature and were periodically subcultured by streaking 0.01 ml. of the preparation over blood agar plates. When the organism was no longer detected by this method, a portion was sedimented by centrifugation and the sediment was cultured. *Nocardia asteroides* remained viable in these drug mixtures for as long as 7 weeks at room temperature (Table 4).

TABLE 4—The Survival of Nocardia asteroides in Antibiotic Infusion Mixtures

	Nocardia strain		
Survival at room temperature (weeks)	A (15,000 *Nocardia*/ml. in 30 cc. mixture containing 90,000 units penicillin, 216 mg. dihydrostreptomycin, 15 cc. nitrofurazone, and 15 cc. saline solution)	G (300,000 *Nocardia*/ml. in 30 cc. saline solution containing 100,000 units penicillin and 250 mg. dihydrostreptomycin)	F (100,000 *Nocardia*/ml. Preparation same as A)
1	5,000*	2×10^6	500,000
2	100	45,000	500
3		9,000	
4	<100†	<100	100
5	100	<100	0
6	0		0
7	0	100	0
8	0	0	0

* Count per milliliter. † No growth when 0.01 ml. of entire mixture cultured but centrifuged sediment was culturally positive.

hospital barn procedure was altered so that nonlactating cows, cows secreting colostrum, and diseased cows were handled separately with proper sanitary precautions.

Following the institution of this regimen in herd F, only a moderate change was observed for 2 months as the parturient cows during this period were those infected under the previous system. Three months after changing the regimen, nocardial infection was markedly reduced and subsequently disappeared from the herd.

Experimental Reproduction of Field Infection.—The isolation of *N. asteroides* from the soil and infusion utensils, the heavy use of therapy in nonlactating cows in these herds and the frequent appearance of clinical disease in recently parturient

Cow 2898 was inoculated with such drug mixtures during the nonlactating period in an effort to reproduce the infection in a manner similar to that occurring under field conditions. This inoculation occurred during the period between her 4th and 5th lactations. The right front quarter was inoculated with 50 ml. of a common therapeutic formula containing 25.0 ml. of 0.2% nitrofurazone, 150,000 units penicillin, and 360 mg. dihydrostreptomycin in enough saline solution to make 50.0 ml. To this mixture, 0.1 ml. of a 96-hour serum broth culture of the herd A isolate was added. The final mixture contained 15,000 *Nocardia* per milliliter. The left rear quarter was inoculated with a similar preparation to which 0.1 ml. of a 96-hour serum broth

culture of *Nocardia* isolated from herd F was added. The final mixture contained 100,000 *Nocardia* per milliliter.

Only transient hyperthermia and moderate swelling immediately after injection were noticed during the remaining 28 days antepartum. Three days postpartum, the rectal temperature was 104 F. and both

Discussion

There is little doubt that the infection is widespread in this country as judged by the number of recently recognized cases. The evidence incriminates faulty aseptic procedures associated with udder infusion techniques. The universal practice of udder infusion by dairymen appears to be the

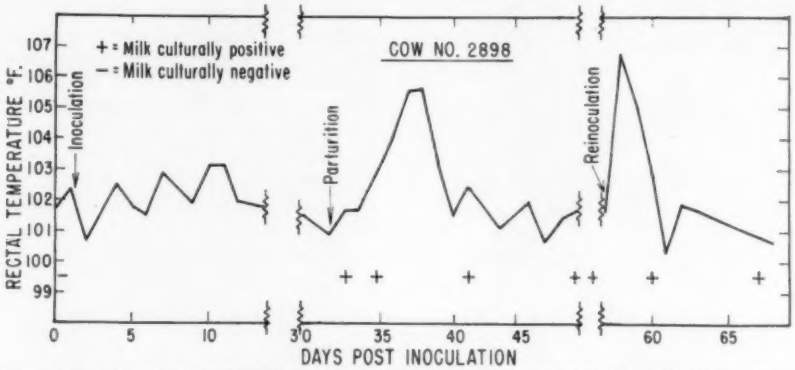

Fig. 1—Clinical and cultural reactions following experimental reproduction of field infection of a cow with Nocardia asteroides.

quarters were firm and shedding visible exudate in the milk. By the 5th day postpartum, the temperature peaked at 105.6 F. and the infected quarters continued to shed visibly altered secretions and were enlarged and firm. Weekly culturing of milk samples drawn from these quarters showed persistence of infection for 3 to 5 months in the right front and left rear quarters, respectively.

The right rear quarter of this cow was inoculated with a broth culture of herd A isolate 1 month after parturition. The temperature again rose and peaked at 106.8 F. 24 hours after inoculation and then gradually receded to normal 4 days later. Mammary involvement resembled that described for the other inoculated quarters of this cow, and milk from this quarter was positive for *N. asteroides* on a majority of cultures made during the 9 months the cow was observed. Occasional negative results were recorded, and one such period of cultural negativity persisted for 5 consecutive weeks, after which the organism was again regularly isolated. The temperature response and the results of cultural examinations of the milk are recorded (Fig. 1).

reason that the organism and susceptible mammary tissue are meeting so frequently. In a recent survey of causative factors of mastitis,[2] it was shown that only about half of the dairymen practiced any kind of asepsis when using intramammary infusion therapy. In herds where *N. asteroides* infection has involved an appreciable segment of the herd, the abolition of syringes, cannulas, and bulk dispensing of therapeutic agents in favor of disposable, single-dose applicators and careful observation of aseptic technique has brought about complete remission of the disease in the herd.

It is unfortunate that the appearance of clinical disease follows so far in the wake of the infecting practice, because the association of one with the other is often disregarded by the herdsman. *Nocardia asteroides* is a relatively slow-growing organism as compared with the more common bacterial pathogens of the mammary gland. The organism apparently needs this quiescent period between lactations to establish itself in the gland when introduced in small numbers. Very few infections have been found during midlactation or in 1st-lactation animals. It is our belief that inapparent

foci of infection become established and expand slowly during the period between lactations, and that at the end of gestation when the gland fills, the limiting adhesions about these foci are disrupted and large numbers of the organism are showered throughout the lobular tree. This results in a massive secondary infection which produces the clinical proportions so often seen shortly after parturition. The process occurs at a time when the cow is in a state of known metabolic stress, which undoubtedly compounds the systemic effects of this dissemination. Such a mechanism would explain the acute clinical syndrome following parturition and also explain why so few cows develop infection during midlactation when exposed to the same management. When the period between lactations is prolonged, the infection may be completely cleared from the mammary tissue. This apparently occurred in an experimental infection which terminated during the subsequent nonlactating period of 5 months' duration.[7]

The appearance of the disease in two 1st-lactation heifers apparently resulted from infection in the heavily contaminated "hospital barns" where they were milked while secreting colostrum. To reduce opportunities for the infection to spread, we have recommended the segregation of diseased cows and the use of disinfectant solutions on the teat ends at the completion of milking in all infected herds.

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SUMMARIO IN INTERLINGUA

Nocardia asteroides Como Pathogeno Mammari in Bestial

Nocardia asteroides esseva isolate ab specimens de terra de corral, ab cannulas de infusion, ab flascos de medicina, e ab partialmente usate mixturas de pharmacos destinate al uso in infusiones intramammari in un establimento ubi le bestial contraheva mastitis nocardial. Le frequente occurrentia del morbo natural in bestial multipare brevemente post le parturition signalava un connexion del infection con le introduction del organismo al tempore del infusion del pharmacos ad in le non-lactifere glandulas mammari.

Nocardia asteroides esseva addite a mixturas de drogas que es frequentemente usate in infusiones intramammari. Esseva trovate que le organismo es viabile in tal mixturas durante 7 septimanas. Le infusion de iste experimentalmente contaminate mixturas de drogas in non-lactifere glandulas mammari produceva un responsa clinic typic del infection in occurrentias natural.

Le institution de technicas appropriatemente aseptic e le substitution de applicatores a uso unic (con doses individual) pro mixturas pluridosal reduceva le incidentia de infectiones in le greges in question.

Growth Rate and Other Signs of Infection in Calves Experimentally Infected with *Cooperia punctata*

Joseph E. Alicata, Ph.D., and Frederick T. Lynd, D.V.M.

SUMMARY

Each of 5 calves, 8 to 13 weeks of age, was given a single oral dose of about 250,000 *Cooperia punctata* infective larvae. Five other calves of approximately the same age and weight were kept as controls.

Signs of the infection included soft feces, intermittent or continued diarrhea, progressive emaciation, reduced feed consumption, weight loss, and listlessness. Individual variation was noticed in the severity of these signs. One calf was found in a moribund condition 3½ weeks postinfection and was killed. Of the remaining 4 calves, 2 appeared to be in poor condition and 2 in fair condition by the end of the 11 weeks of the trial.

The average weekly weight gain of the 4 remaining infected calves for the 11-week-period was 5.9 lb. as compared with 9.41 lb. for the 5 control calves.

INTRODUCTION

Cooperia punctata is a common and widely distributed parasite of calves. Its incidence has been reported to be 95% in Hawaii,[1] 91% in southeastern United States,[7] about 80% in Texas,[4] 72% in Australia,[9] and 55% in Puerto Rico.[2]

In Hawaii, *Cooperia* infection has been found most prevalent in beef calves at 8 months of age and in dairy calves from 4 to 8 months of age. Unthriftiness has also been associated with a heavy infection with this parasite.[1]

In view of these reports and the limited information on the pathogenicity of *Cooperia punctata*,[3, 5, 6, 8] it was desirable to conduct further observations on the clinical signs, growth rate, and lesions produced in animals by this parasite. Only the observations on signs of infection, including the effect on growth rate, are reported here.

Received for publication Oct. 31, 1960.

From the Department of Parasitology, Hawaii Agricultural Experiment Station, University of Hawaii, Honolulu, and the Bureau of Veterinary Laboratory, Department of Agriculture and Conservation, Honolulu. Published with the approval of the director of the Hawaii Agricultural Experiment Station as Technical Paper No. 505.

This investigation was conducted as a part of the Western Regional Research Project W-35, Internal Parasites of Ruminants.

Materials and Methods

Ten bull calves were obtained at 4 days of age from a local dairy. Five were experimentally infected with approximately 250,000 infective *Cooperia* larvae, given orally in 1 dose, when the calves were from 8 to 13 weeks of age. The other 5 calves, approximately of the same age and weight, served as noninfected controls. The calves were kept in individual stalls with concrete floors covered with a layer of wood shavings. The floors were thoroughly cleaned twice a week. All calves were fed milk twice a day until they were about 6 weeks of age. Recommended amounts of a commercial grain mixture and chopped alfalfa hay were offered to the animals when they were about 10 days old and continued after they were weaned. The calves were recorded in 2 groups as follows: group A (control), calves 111, 112, 113, 114, and 115; group B (infected), calves 116, 117, 118, 119, and 120. All calves were weighed and examined for parasites once every week. Egg counts, using the direct centrifugal flotation-brine method, were also made from the feces of the infected animals. Daily records of the consistency of the feces and of feed consumption were maintained.

The experiment was terminated at the 11th postinfection week, when the parasite egg-count was comparatively low. At necropsy, the number of parasites present was determined from aliquots of the intestinal contents.

Results and Discussion

Parasite Egg Count.—Cooperid eggs were found in the feces of calves of group B when first examined 12 days after experimental infection. The highest egg count was recorded 17 days postinfection (range, 5,320 to 8,850; av. 6,624 per gram of feces). In each of the subsequent 9 weeks, the average count was as follows: 2,190, 1,425, 1,346, 930, 1,022, 931, 549, 33, and 5, respectively. There was no significant difference in the egg counts recorded for the infected animals. The control calves re-

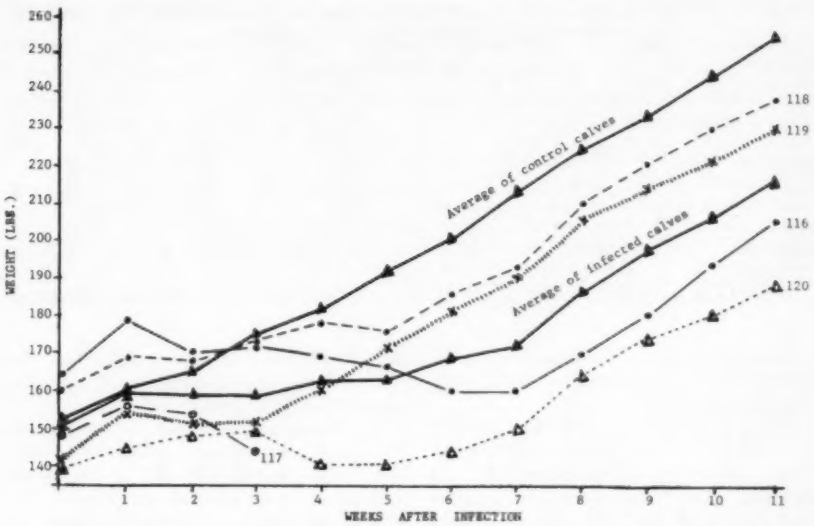

Fig. 1—Individual weekly body weight of calves infected experimentally with Cooperia larvae. Average weights of infected and control calves are indicated by solid lines.

mained consistently negative for cooperid eggs throughout the experiment.

Weight Records.—The average weight gain or loss is shown graphically (Fig. 1). The total weight of the calves of group B at the time of infection was 753 lb., and that of the control calves (group A) was 760 lb. The control calves made uniform and consistent gains and appeared in good condition (Fig. 2). By the end of the 11th week, they had made an average weekly weight gain of 9.41 lb. (Table 1). The infected calves (group B) made comparative gains during the 1st week of infection, but lost weight in the subsequent 2 weeks (Fig. 1). Three and a half weeks after infection, calf 117 had become emaciated and listless and was killed. The remaining calves of group B began making weight gains during the 7th to 8th week of infection. The average weekly gain for the in-

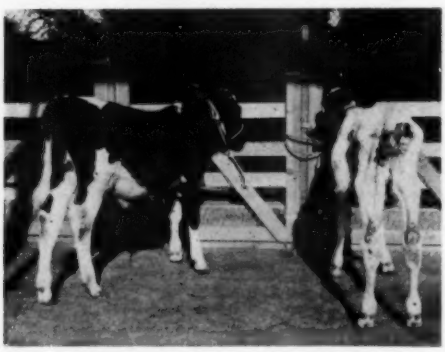

Fig. 2—Physical appearance of control calf 111 (left) and calf 116 (right) 5 weeks after experimental infection with approximately 250,000 infective Cooperia punctata larvae. Notice evidence of emaciation and diarrhea in calf 116.

TABLE 1—Average Weekly Weight Gain (lb.) of Calves Experimentally Infected with Cooperia punctata (11-Week Period)

Calf No.	Original weight	Final weight	Total gain	Average weekly gain
GROUP A (CONTROL)				
111	183	284	101	9.19
112	135	234	99	9.00
113	152	260	108	9.81
114	148	256	108	9.81
115	142	244	102	9.27
Totals	760	1,178	518	47.09
Average	152	235.6	101.6	9.41
GROUP B (INFECTED)				
116	164	206	42	3.81
117	148	144*	....	.....
118	160	238	78	7.09
119	141	232	91	8.27
120	140	189	49	4.45
Totals	753	1,009	260	23.62
Average	150.6	201.8	65.0	5.90

* Final weight 3 weeks after infection. This calf, in a moribund condition, was killed 2 days after the last weighing.

TABLE 2—Abnormal Fecal Consistency and Feed Consumption Observed Each Week Following Experimental Infection with Cooperia punctata

Week of infection	Calf Number				
	116	117	118	119	120
1st	.....	.....	.....	.....	.....
2nd	.....	S	.....	.....	.....
3rd	S, D	D, ½–¼ F	S	S	S
4th	S, D, ½–¼ F	(killed)	S, ½ F	D, ½ F	D, ½ F
5th	S, D, ½–¼ F	.....	S, ½ F	S	S, ½ F
6th	S, D, ½–¼ F	.....	S, D, ½ F	S, D	D, ½ F
7th	S, D, ½–¼ F	.....	S	.....	D, ½ F
8th	S, D, ½–¼ F	.....	S	S	S
9th	S, D, ½ F	.....	.....	.....	D
10th	.....	.....	.....	.....	D
11th	.....	.....	.....	.....	½–¼ F

S = soft feces; D = fluid feces; F = quantity of daily ration consumed when consumption was reduced.

fected calves during the 11 weeks of observation was 5.9 lb., as compared with 9.41 lb. for the control calves (Table 1).

The record on weight gain of the infected animals (Table 1) indicates considerable individual difference in susceptibility to this parasite. The most prominent signs were manifested by calf 117 which became moribund in 3½ weeks. Calves 116 and 120 made poor weekly gains (av. 3.81 and 4.45 lb., respectively). Calves 118 and 119 were least affected, and their weekly gains (7.09 and 8.27 lb., respectively) were not far below the average gain of the control group (9.41 lb.).

Feed Consumption and Fecal Consistency.—None of the control calves were ever inappetent. On the contrary, all the infected calves had varying degrees of reduced feed consumption. Soft feces or diarrhea appeared by the 2nd to 3rd week of infection (Table 2). Calf 116 ate about half of the daily ration between the 4th and 9th week, but his appetite improved during the last 2 weeks. During the period of anorexia, the feces were either soft or fluid. Calf 117 consumed little of the daily ration and diarrhea was evident during the 3rd week of infection. A few days later, this animal was killed. Varying degrees of anorexia and scouring were noticed in calves 118 and 119. Calf 120 consumed about half of its normal ration during the 4th to the 9th week of infection, and the feces were either soft or fluid during most of the period. Of the noninfected control calves, 112, 113, and 115 had soft feces for 1 or 2 days during the 7th week of the experiment, but there was no evidence of parasites.

Findings at Necropsy.—The number of *C. punctata* worms recovered from the small intestine of calf 117 at necropsy was estimated to be 35,000. However, the following numbers of *Cooperia* were found in the remaining 4 calves at necropsy 11 weeks after infection: calf 116, 1,020; calf 118, 740; calf 119, 560; calf 120, 1,580. No *Cooperia* or other worms were found in 3 of the control animals killed at the same time.

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SUMMARIO IN INTERLINGUA

Retardo de Crescentia e Altere Signos de Infection in Vitellos Experimentalmente Inficite con *Cooperia punctata*

Cinque vitellos, de 8 a 13 septimanas de etate, recipeva cata un sol oral de circa 250.000 larvas infective de *Cooperia punctata*. Cinque altere vitellos de approximativemente le mesme etates e pesos esseva usate como animales de controlo.

Le signos del infection includeva feces molle, diarrhea intermittente o continue, emaciation progressive, reducite consumo de alimento, perdita de peso, e apathia. Esseva notate variationes individual in le severitate de iste signos. Un vitello esseva trovate in stato moribund 3½ septimanas post le infection. Illo esseva occidite. Inter le altere 4 vitellos, 2 pareva esser in condition mal e 2 in condition mediocre verso le fin del periodo experimental de 11 septimanas.

Le ganio de peso medie per septimana in le 4 supervivente vitellos pro le periodo de 11 septimanas esseva 5,9 lb, comparate con 9,41 lb pro le 5 vitellos de controlo.

A Critical Evaluation of Bephenium Hydroxynaphthoate as an Anthelmintic in 8 Yearling Hereford Calves

Amin M. Eisa, B.V.Sc., M.S., and Robert Rubin, D.V.M., M.S.

SUMMARY

Eight parasitized yearling Hereford heifers were used to study the anthelmintic efficacy of a commercial preparation containing 90% bephenium hydroxynaphthoate. Four of the calves were given the preparation at the rate of 250 mg. per kilogram of body weight. The remaining 4 calves served as untreated controls. The efficacy of bephenium hydroxynaphthoate in this study was based on a comparison of the total number of worms recovered from the treated calves with that recovered from the controls. On this basis, there was 85% reduction for *Ostertagia ostertagi*, 87% reduction for *Trichostrongylus axei*, 100% reduction for *Nematodirus helvetianus*, 99% for *Cooperia oncophora*, 100% for *Oesophagostomum radiatum*, and 99% for *Chabertia ovina*.

No harmful effects were noticed in the 4 experimental calves, each of which was given a single dose of the preparation at the rate of 250 mg. per kilogram of body weight. In addition, there were no macroscopic or microscopic lesions noticed in the gastric and intestinal mucosae of the treated calves that could be attributed to bephenium hydroxynaphthoate.

Bephenium hydroxynaphthoate appears to possess strong anthelmintic activity in the abomasum, small intestine, and large intestine. This feature should greatly enhance its value as a broad-spectrum anthelmintic for cattle.

Received for publication Sept. 26, 1960.

From the College of Veterinary Medicine, Colorado State University, Fort Collins.

This paper is based on a thesis submitted by the senior author to the Graduate School of Colorado State University in partial fulfillment of the requirements for the M.S. degree.

This research was supported by a grant from Burroughs Wellcome & Co., Tuckahoe, N.Y.

INTRODUCTION

A recent encouraging development in the search for better anthelmintics has been the finding of a new series of chemical compounds designated by the generic name bephenium.[3] Members of this series of drugs have been shown to have marked activity against a number of nematodes of sheep,[4,6-8,12] dogs,[1,2] and human beings.[5,9,13]

Rubin[10] investigated the effect of bephenium embonate on egg production of *Nematodirus helvetianus* and other gastrointestinal nematodes in cattle, and observed that a single dose of 250 mg. of the commercial preparation* per kilogram of body weight resulted in marked reduction, not only of *Nematodirus* egg counts but also of other trichostrongylid eggs. He also obtained good results using bephenium hydroxynaphthoate* in a herd of cattle that was similarly parasitized.

British workers have found bephenium hydroxynaphthoate has a higher efficacy against a broader spectrum of parasites of cattle and sheep than the embonate salt. Its toxicity is low, only moderately greater than that of the embonate and considerably lower than that of the halide salts.**

The following report is a critical study of the anthelmintic activity of bephenium hydroxynaphthoate in a group of 8 parasitized yearling Hereford calves.

Materials and Methods

Clinical parasitism was diagnosed in a herd of approximately 100 cattle located on a farm northwest of Fort Collins, Colo. On May 15, 1959, 8 of the yearling calves were obtained from this herd in order to conduct a critical evaluation of bephe-

* Franten (bephenium hydroxynaphthoate), produced by Burroughs Wellcome & Co., Tuckahoe, N.Y. This drug is commercial known as Franten Mark III, and contains 90% bephenium hydroxynaphthoate (super-fine powder). The remaining 10% of ingredients are pharmacologically inactive.

** Dr. J. R. Wood, vice president and director of research, Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.: Personal communication, April 14, 1959.

nium hydroxynaphthoate on the gastrointestinal helminths they harbored.

Rectal fecal material was collected from the 8 calves, usually daily, beginning a week prior to treatment and continuing for each calf until the date of its necropsy. Recovery of eggs from the fecal material was by means of a simple, yet seemingly accurate and reliable, flotation method with a saturated solution of sodium chloride.

In an effort to allot the calves to 2 equally parasitized groups, it was assumed that the number of eggs passed in the feces correlated with the number of worms in the gastrointestinal tract of each animal. The calves with the highest average eggs per gram (e.p.g.) counts were paired, the calves with the next highest average were also paired, and so forth, resulting in 4 pairs of calves. On the basis of these egg counts, it was assumed that each pair consisted of 2 calves with comparable numbers of worms. Next, using random selection, 1 member of each pair was placed in either lot A or lot B. Subsequent random selection determined that those in lot A would be treated and those in lot B would serve as untreated controls.

Each calf to be treated was weighed within 24 hours prior to treatment, and a single dose of the commercial preparation, 250 mg./kg. of body weight (equivalent to 225 mg. of bephenium hydroxynaphthoate per kilogram), was orally administered in 0.5-oz. gelatin capsules with a balling gun.

The weights for these calves and the calculated amount of drug given to each calf are shown (Table 1).

TABLE 1—Weights of the 4 Calves to be Treated, and the Amount of Bephenium Hydroxynaphthoate Given to Each Based on a Dosage of 225 mg. of Commercial Preparation per Kilogram of Body Weight

Calf No.	Weight (kg.)	Amount of commercial preparation (Gm.)
1342	129.1	32.25 (equivalent to 29.1 Gm. BHN*)
1343	110.4	27.6 (equivalent to 24.8 Gm. BHN*)
1344	143.6	35.9 (equivalent to 32.3 Gm. BHN*)
1349	96.8	24.2 (equivalent to 21.8 Gm. BHN*)

* BHN = bephenium hydroxynaphthoate.

Ninety-six hours following treatment, each treated calf was euthanatized and necropsied, and a day later its untreated mate was also necropsied.

The gastrointestinal tract was removed intact and placed on wrapping paper to prevent contamination. The abomasum, small intestine, and large intestine were opened, and their contents were washed separately, using a standard procedure. The mucosa of each organ was also thoroughly washed and scraped in an effort to obtain as many of the parasites as possible.

Boiling 10% formalin was used as a preservative because it killed and fixed the worms in an extended state, making later identification easier.

Since microscopic changes might be present in the tissues but not observable macroscopically, representative tissues along the gastrointestinal tract were collected and preserved in 10% formalin for later examination.

The efficacy of bephenium hydroxynaphthoate in this experiment was evaluated for each of the genera present by comparing the total number of worms remaining after treatment with the total recovered from the untreated calves.

The worms from the large intestine were big enough to be seen readily and removed from the sediment. Total numbers were determined by direct counting. For the worms in the abomasum and small intestine, total numbers were determined by the usual dilution technique. Usually, 3 samples from the diluted contents from each organ were withdrawn; however, in the case of the treated calves, a number of samples contained no worms. In these instances, 6 to 10 samples were examined in order to detect any worms which were so few in number that they were not recoverable in each individual sample.

Each sample was examined with the aid of a dissecting microscope. The worms were collected and separated into different genera. Worms from the different genera were placed on microscope slides and cleared in either lactophenol or polyvinyl alcohol.[11] These were then examined under a calibrated microscope for species determination.

Results

Effect of Bephenium Hydroxynaphthoate on the Clinical Condition of the 4 Calves.—There was no detrimental effect noticed following the oral administration of bephenium hydroxynaphthoate to each of 4 calves at the dosage rate of 225 mg./kg. of body weight. For 4 days following treatment, the appetite remained normal, the body temperature of each calf was within normal range (101.2 to 102 F.), and the animals were not depressed.

In 4 untreated control calves, varying degrees of diarrhea persisted throughout the experimental period. However, the drug had the apparent effect of changing the consistency of the feces in 3 of the 4 treated calves from watery to normal. The effect of treatment on the fecal consistency of the 4th calf was not clearly apparent. Its feces, which were also diarrhetic at the time of treatment, varied between normal and diarrhetic during the 4 days following treatment. Also, the feces of this calf had an unusual fetid odor and were darker.

Effect of Bephenium Hydroxynaphthoate on the Number of Eggs per Gram of Feces.—There was a marked reduction in the number of trichostrongylid eggs per gram of feces within the first 24 hours following treatment in each of the 4 calves.

During the next 3 days, this reduced count remained essentially unchanged. The average trichostrongylid egg count for these calves on the day of treatment was 351. Twenty-four hours after treatment, it was reduced to 46. Ninety-six hours following treatment, the count was 34 eggs per gram.

The per cent reduction in the egg counts ranged from 83 for calf 1343 to 93 for calf 1349, with an over-all average of 90% for all treated calves (Table 2).

TABLE 2—Trichostrongylid Eggs per Gram of Feces of 4 Calves from the Day of Treatment with Bephenium Hydroxynaphthoate to the Day of Necropsy and the Per Cent Reduction for Each Calf During This Interval

Calf No.	No. of eggs per gram of feces at treatment time	Post-treatment (hr.)				Per cent reduction
		24	48	72	96*	
1342	314	20	24	20	28	91
1343	246	46	25	45	43	83
1344	256	40	23	41	34	87
1349	586	76	80	31	32	93

* At time of necropsy.

In 3 of 4 treated calves, the number of *N. helvetianus* eggs per gram of feces was reduced to zero within the first 24 hours following treatment. In the 4th calf, the number of *Nematodirus* eggs per gram of feces was reduced from 10 to 2 during the first 24 hours following treatment, and was further reduced to zero within the next 24 hours. Thus, in all 4 instances, there was a 100% reduction (Table 3).

TABLE 3—Nematodirus Eggs per Gram of Feces of 4 Calves from the Day of Treatment with Bephenium Hydroxynaphthoate to the Day of Necropsy and the Per Cent Reduction for Each Calf During This Interval

Calf No.	No. of eggs per gram of feces at treatment time	Post-treatment (hr.)				Per cent reduction
		24	48	72	96*	
1342	10	0	0	0	0	100
1343	1	0	0	0	0	100
1344	10	2	0	0	0	100
1349	12	0	0	0	0	100

* At time of necropsy.

Fecal examinations also showed eggs of *Trichuris sp.* in 2 of the 8 calves. This incidence was considered to be too low to be useful in evaluating bephenium hydroxynaphthoate against this parasite.

Tables 4 and 5 show the daily egg counts for the untreated control of each pair of calves for the same time interval shown for the treated calves (Tables 2, 3). The average trichostrongylid and *Nematodirus* eggs per gram in the untreated control calves remained essentially constant or increased during this period.

TABLE 4—Trichostrongylid Eggs per Gram of Feces of 4 Control Calves for the Same Interval as Shown for the Treated Calves (Table 2)

Calf No.	No. of eggs per gram of feces on day of treatment*	Post-treatment (hours)				
		24	48	72	96	120**
1345	146	153	148	166	147	205
1346	130	247	278	361	286	296
1347	129	115	123	164	159	148
1348	295	160	323	383	182	201
Average	175	169	218	269	194	213

* Principals treated with bephenium hydroxynaphthoate.
** Day of necropsy.

TABLE 5—Nematodirus Eggs per Gram of Feces of 4 Control Calves for the Same Interval as Shown for Treated Calves (Table 3)

Calf No.	No. of eggs per gram of feces on day of treatment*	Post-treatment (hr.)				
		24	48	72	96	120**
1345	1	8	0	3	0	2
1346	5	10	5	5	3	6
1347	0	0	0	0	0	0
1348	2	4	1	5	2	4
Average	2	6	2	3	1	3

* Principals treated with bephenium hydroxynaphthoate.
** Day of necropsy.

Effect of Bephenium Hydroxynaphthoate on the Worm Population.—At necropsy, *Ostertagia ostertagi* and *Trichostrongylus axei* were found in the abomasum of each of these 8 calves. A comparison was made of the number of worms for each species in each of the 4 treated calves with the number of worms in their particular controls (Table 6). The reduction for *O. ostertagi* varied between 75 and 90% in the 4 pairs. On the basis of the total number of worms in each group, the per cent reduction was 85. The per cent reduction for *T. axei* varied between 73 and 93, with a group average of 87%.

In the small intestines of these calves there were *N. helvetianus* and *Cooperia onchophora*. The reduction for *N. helvetianus* was 100% in the 4 treated calves. The per cent reduction for *C. onchophora* varied between 95 and 100, with a group average of 99% (Table 6).

The 3 species of worms encountered in the large intestines of these 8 calves were *Oesophagostomum radiatum*, *Chabertia ovina*, and *Trichuris sp.* As previously mentioned, the incidence of *Truchuris sp.* was too low to be considered in this study.

Reduction in *Oe. radiatum* was 100% in the 4 treated calves. The reduction in *C. ovina* varied between 97 and 100%, with a group average of 99% (Table 6).

These data were submitted to statistical analysis using the *chi* square test for goodness of fit. For each genus, the reduction in numbers of worms by treatment was highly significant at the 0.1% level.

Pathologic Findings.—There were no grossly discernible differences in the gastrointestinal tracts of the treated calves and their controls. This evidence is suggestive that bephenium hydroxynaphthoate did not produce lesions which persisted for the 4 days between the time of medication and the time of necropsy.

Although only a very small proportion of the entire gastric and intestinal mucosae was examined microscopically, it appeared that there were more parasites in the tissues from the control calves than there were in those from the treated calves. The reaction to the presence of the parasite was the same, regardless of whether the animal had been treated.

TABLE 6—Comparison of the Numbers of Worms Recovered from Each of the 4 Treated Calves with Those Recovered from the Untreated Controls

Pair No.	Calf No.*	Abomasum				Small intestine				Large intestine			
		Ostertagia ostertagi	Per cent reduction	*Trichostrongylus axei*	Per cent reduction	*Nematodirus helvetianus*	Per cent reduction	*Cooperia oncophora*	Per cent reduction	*Oesophagostomum radiatum*	Per cent reduction	*Chabertia ovina*	Per cent reduction
1	1348(C)	1,332	88	927	93	305	100	1,175	98	308	100	62	97
	1349(T)	163		69		0		22		0		2	
2	1345(C)	2,377	90	1,740	90	261	100	3,132	100	263	100	28	100
	1342(T)	231		172		0		0		0		0	
3	1346(C)	1,148	78	626	73	130	100	696	95	161	100	16	100
	1344(T)	247		166		0		36		0		0	
4	1347(C)	1,218	75	924	87	0	100	610	97	291	100	28	100
	1343(T)	300		125		0		18		0		0	
Total of 4 calves	(C)	6,075	85	4,217	87	696	100	5,613	99	1,023	100	134	99
	(T)	1,141		532		0		76		0		2	

* T = Treated with bephenium hydroxynaphthoate; C = untreated control.

Discussion

Although the mode of action of bephenium hydroxynaphthoate is not known, the data reported here suggest that the drug exerts much of its anthelmintic effect within the first 24 hours following administration. This rapid action was evidenced in the treated animals by marked reduction in the number of eggs per gram of feces during the first 24 hours after treatment. Since necropsy findings showed that there was a reduction in the number of worms in treated animals as compared with that of the untreated controls, it is concluded that the reduction in the number of eggs was a result of the death of the worms and not from the suppression of egg production.

Rapid anthelmintic activity was also reported by Burrows,[2] who found that the stool specimens passed from dogs 3 hours or more after administration of the drug contained hookworms, and in several animals all or almost all of the hookworms were passed in less than 7 hours. He stated that of all the hookworms killed by these

compounds, 97.3% were eliminated during the first 24 hours after dosing.

On the basis of this experiment, the authors can conclude only that bephenium hydroxynaphthoate is highly effective against those parasites harbored by the calves used in this study. Further investigations with bephenium hydroxynaphthoate against a greater variety of gastrointestinal nematodes of cattle are needed before the entire spectrum of activity of the drug is determined. The fact that other investigators [4,6-8,12] have found the drug to be active against many of the gastrointestinal parasites of sheep should make one optimistic that the range of activity in cattle extends beyond the parasites reported in this study.

The proper usage of any anthelmintic, including bephenium hydroxynaphthoate, should be such that only the minimal amount of drug needed to achieve maximum efficacy should be used. As stated earlier, the dosage rate of 250 mg. per kilogram of body weight of the drug preparation used in this experiment was based on experiences with its use in sheep. It is entirely possible that this is not the optimum dosage for cattle, and it will require further experimentation to determine if there is a better one. Also, no attempt was made in this limited study to determine whether the efficacy of the drug could be increased by giving it in divided doses rather than in a single dose, and this must also be determined in order to arrive at the best possible usage of it.

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SUMMARIO IN INTERLINGUA

Un Evaluation Critic de Hydroxynaphthoato de Bephenium, Usate Como Anthelmintica in Octo Vitellos Hereford del Prime Anno

Octo parasitisate vaccas Hereford del prime anno esseva usate pro studiar le efficacia anthelmintic de un preparato commercial continente 90% de hydroxynaphthoato de bephenium. Quatro del animales recipeva le preparato in un dosage de 250 mg per kg de peso corporee. Le altere 4 serviva como nontractate subjectos de controlo. Le efficacia de hydroxynaphthoato de bephenium esseva estimate a base del comparation del numero total de vermes recuperate ab le tractate animales con illo recuperate ab le animales de controlo. Secundo iste criterio il occurreva un reduction per 85% in *Ostertagia ostertagi*, per 87% in *Trichostrongylus axei*, per 100% in *Nematodirus helvetianus*, per 99% in *Cooperia ancophora*, per 100% in *Oesophagostomum radiatum*, e per 99% in *Chabertia ovina*.

Nulle adverse effectos esseva notate in le 4 animales experimental que habeva recipite le preparato in le supra-indicate dosage de 250 mg per kg de peso corporee. In plus, esseva notate in le mucosas gastric e intestinal del tractate animales nulle lesiones macro- o microscopic de character attribuibile a hydroxynaphthoato de bephenium.

Hydroxynaphthoate de bephenium pare posseder un forte activitate anthelmintic in le abomaso, le intestino tenue, e intestino crasse. Iste characteristica servi sin dubita a augmentar grandemente su valor como anthelmintico a large spectro in le tractamento de bestial.

Vesicular Stomatitis—Patterns of Complement-Fixing and Serum-Neutralizing Antibodies in Serum of Convalescent Cattle and Horses

J. N. Geleta, D.V.M., M.S., and A. A. Holbrook, D.V.M.

SUMMARY

The complement-fixing and serum-neutralizing antibody patterns were determined in serum samples serially collected from 22 cattle and 8 horses that had been experimentally infected with New Jersey and Indiana types of vesicular stomatitis virus.

Serum tested for antibody by direct complement-fixation had specific and reproducible titers. In both cattle and horses, the diagnostic titers appeared 6 to 8 days after inoculation and reached a peak in 9 to 16 days. Thereafter, they gradually declined and disappeared in 50 to 110 days.

Serum-neutralizing titers were demonstrated at 6 to 8 days postinoculation and increased to relatively high levels within 4 to 5 weeks. The titers of the neutralizing antibodies remained high but fluctuated irregularly in the range of less than 2 logs for the duration of the test.

Most of the recovered animals, in which the complement-fixing titer had disappeared although the serum-neutralizing titer remained high, were susceptible to challenge inoculations with a homologous-type virus. In both susceptible and immune animals, the titers rose rapidly after challenge.

INTRODUCTION

Infection with vesicular stomatitis (VS) in cattle and horses results in transient immunity and the occurrence of specific antibodies in animal serums. The early detection and measurement of these antibodies by complement-fixation (CF) and serum-neutralization (SN) procedures are valuable in differential diagnosis of vesicular diseases.

Rice and McKercher,[6] using the direct CF method, studied the antibody activity in serum of horses convalescent from the New Jersey and Indiana types of vesicular stomatitis virus (VSV). They reported that complement-fixing antibodies were detected 6 to 8 days after inoculation. In 10 days to 2 weeks, the titers reached a peak that remained relatively constant for 7 to 10 days and then declined slowly. In demonstrating antibody activity in serum of convalescent cattle, these workers used an indirect CF method. In the indirect tests, the time for antibody appearance in cattle serums coincided with that demonstrated by direct CF tests in horse serum.

Sorensen *et al.*,[7] using the SN test, recently investigated the activity and persistence of SN antibodies in cattle naturally and experimentally infected with the New Jersey type of VSV. These workers reported that the SN antibody titers reached a peak in about 2 months and then fluctuated in cycles for several months with peaks at 8, 14, and 23 months and marked lows between.

In continuing studies at this laboratory, CF and SN tests were conducted on serum samples from animals experimentally infected with the 2 VSV types. This report compares the specificity, appearance, persistence, and titers of CF and SN antibodies in cattle and horses exposed to live virus. Variations in the immunologic responses of animals are discussed.

Materials and Methods

The animals used were cattle from the Animal Disease Station and the Animal Husbandry Unit of the Agricultural Research Center, Beltsville, Md., and horses from premises known to be free of VS infection. The cattle were Holstein-Friesians, Guernseys, and Jerseys 2 to 7 years of age. Twenty-two cattle and 8 horses were inoculated intradermolingually (IDL)—12 cattle and 4 horses with the New Jersey type of VSV and 10 cattle and 4 horses with the Indiana type.

Received for publication Aug. 3, 1960.

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The authors thank Dr. L. O. Mott, virological investigations leader, for assistance and valuable advice.

The inoculum was prepared from virus-infected bovine or equine tongue epithelium ground in a mortar and diluted in saline solution, buffered at pH 7.6, to make a 5% suspension. After centrifuging at 2,000 r.p.m. for 10 minutes in an International No. 2 centrifuge, the inoculum was injected IDL, using 10 to 15 ml. per animal. Penicillin (2,500 I.U./ml.) and streptomycin (2.5 mg./ml.) were added to prevent bacterial contamination. Challenge inoculations, using similar viral preparations, were made on 5 recovered animals several months after the primary infection.

The animals inoculated with the 2 VSV types were isolated in different screened barns under strict quarantine (all personnel were required to wear laboratory clothing in the quarantine area and take a decontaminating shower before leaving). One month after exposure, the animals were moved to holding barns or field pens and later to pasture lots. Blood samples were taken before inoculation and at various intervals afterward—as long as 13 months for most animals and 48 months for a few.

COMPLEMENT-FIXATION TEST

Antigens.—The antigens used in both CF and SN tests were prepared from egg-adapted New Jersey and Indiana types of VSV as follows: 0.15 ml. of virus-laden allantoic fluid, treated with penicillin (1,000 I.U./ml.) and streptomycin (1.0 mg./ml.), was injected into the allantoic cavities of 8-day embryonating chicken eggs. The injection was made through a hole in the center of the air space with a ¾-inch, 26-gauge needle as previously described.[2] The infected eggs were reincubated at 35 C. All embryos that died between 16 and 28 hours postinoculation were chilled overnight at 4 C. Allantoic fluids were vacuum aspirated with a 15-gauge needle attached by a rubber hose to an iced filter flask. Chorioallantoic membranes were then aseptically collected. One part of pooled chorioallantoic membranes and 4 parts of infected allantoic fluid were homogenized for 4 minutes in a Waring Blendor. This stock viral antigen was dispensed in small vials and stored at −60 C. The antigens were closely comparable on CF titrations (titer was 1:128 or higher) and retained their original titers for at least 5 months. The LD_{50} dose for 0.15 ml. of inoculum was $10^{-8.5}$ when tested in 8-day chicken embryos.

The control antigen for nonspecific CF activity was made in the same manner. One part of chorioallantoic membranes from noninfected 9-day embryonating eggs was blended with 4 parts of normal allantoic fluids.

Diluent.—The diluent used in the CF tests was veronal buffer, prepared as described by Brooksby [1] but without calcium and the magnesium chloride.

Sensitized Ovine Erythrocytes.—Defibrinated blood from a normal sheep was preserved in an equal volume of modified Alsever's solution (ARS formula—0.42 Gm. of sodium chloride, 1.2 Gm. of sodium citrate, and 2.05 Gm. of dextrose for each 100 ml. of solution) and stored at 4 C. A portion of this suspension was removed daily, washed 3 times in saline solution, and resuspended to a 3% concentration in veronal saline solution for the CF tests. Final optical density to 0.34 was obtained using a Coleman junior spectrophotometer. These cells were then sensitized with 2 units of hemolysin as described by Jenney *et al.*[3]

Complement.—Complement from guinea pigs was prepared and titrated as described by Jenney *et al.*,[3] using 1.9 units throughout the test.

Antigen Titrations.—All antigens were thawed rapidly and centrifuged in an International No. 2 centrifuge at 2,000 r.p.m. for 10 minutes. The stock viral antigens were titrated by serial twofold dilutions against standard 1:5 hyperimmune guinea pig serum. The antigen titers were 1:128 or higher. For the CF test, the stock membrane antigen was diluted 1:5, which contained 4 units as determined by the method of Kolmer *et al.*[4] The control antigen was also diluted 1:5.

Serums.—Serum samples were obtained from all animals before and after inoculation. They were stored at −60 C. or preserved with phenol (0.5%) and held at 4 C. All samples tested were diluted 1:5 in veronal-buffered saline solution and inactivated by heat. The cattle serum samples were heated for 30 minutes at 56 C., whereas, the horse serum was heated to 58 C. for 30 minutes. Complement-fixation tests were conducted simultaneously on all serum samples obtained from one animal.

Direct Method.—The CF technique for serum titration described by Jenney *et al.*[3] was used throughout the experiment.

A 1:5 serum dilution was used to start the dilution series. Twofold dilutions of each tested serum were made in veronal-buffered saline solution and distributed in 0.25-ml. amounts into 3 sets of tubes. To each tube, 0.25 ml. of complement and 0.25 ml. of 1:5 dilution of stock antigen were then added. The 1st set of tubes received

the homologous antigen, the 2nd set the heterologous antigen, and the 3rd set the normal antigen. The resulting mixture of serum, complement, and antigen was thoroughly shaken and incubated in a water bath at 37 C. Following 2 hours' fixation, 0.5 ml. of the sensitized sheep red blood cells was added to each tube, and the incubation continued at 37 C. for an additional 30 minutes.

Control of antigens, test serums, known New Jersey and Indiana immune serums, complement, and sensitized blood cells were included in all serum titration or specificity tests. The CF test was read visually and the results recorded from minus (complete hemolysis) to 4+ (complete absence of hemolysis). The end points were selected as the highest dilution of serum resulting in more than 2+ fixation.

SERUM-NEUTRALIZATION TEST

Serum-neutralization tests were performed by mixing serial tenfold dilutions of homologous egg-membrane virus suspension with equal volumes of a 1:5 dilution of each type of inactivated serum. Antibiotics were added to the mixtures to make a final concentration of 2,500 I.U./ml. of penicillin and 2.5 mg./ml. of streptomycin. After incubation in a water bath at 25 C. for 30 minutes, the mixtures were removed and placed in an ice-water bath. Five 8-day embryonating chicken eggs were each inoculated in the allantoic cavity with 0.15-ml. amounts of each serum-virus mixture and incubated further at 35 C. for 4 days. The inoculated eggs were candled daily and deaths of all embryos were recorded. All serum samples obtained from one animal were tested at the same time.

The LD_{50} end-point was calculated by the method of Reed and Muench.[5] Virus-neutralizing activities of the samples were expressed as neutralizing indexes which represent the difference in the titer of virus in the presence of normal preinoculation serum and in the presence of the immune postinoculation serum. A serum was regarded positive if it had a neutralizing index of 2.5 or greater.

Results

Both horses and cattle were readily infected with either type of VSV and displayed characteristic signs of the disease. In most of these animals, there was typical vesiculation 24 hours after exposure. The CF and SN antibodies were observed in the serum from all infected animals.

ANTIBODIES IN CATTLE SERUMS

Complement-Fixing Antibody.—The results of CF tests using serums from cattle infected with New Jersey or Indiana types of VSV are shown (Fig. 1). The course of antibody appearance and the changes in titers of serum at periodic intervals are presented.

The individual CF titers among New Jersey-VS convalescent cattle differed considerably in the levels but were uniform in persistence of detectable antibody. For those infected with New Jersey-VS virus, there were negative results in the tests with preinoculation serum and postinoculation serum obtained within the first 5 days after exposure. Type-specific antibodies were first detected 6 or 7 days postinoculation. A rapid rise in the CF titers was observed in subsequent blood samples and the maximal titers were demonstrated in 7 of the 9 animals 9 to 15 days postinoculation. Cows 3085 and 2905, inoculated at the same time, had irregular reactions and reached the highest antibody level at 6 weeks after exposure. Antibody values for these 2 cows were not included in the average titer curve but were recorded individually. After attaining the peak, the antibody levels declined gradually until they became undetectable at 90 to 110 days postinoculation.

The time of response of Indiana-VS convalescent cattle was variable (Fig. 1) but the serum titers of individual animals were similar. The CF antibody levels of 2 cows that had developed delayed vesiculation were somewhat lower and developed more slowly than in the other animals in this group. In 5 of 8 convalescent cattle, the CF activity was demonstrated on the 6th day and in the 3 other cows on the 8th day postinoculation. A rapid increase of the average serum titer was observed on the 9th to 11th day and the highest elevation in titer occurred within 12 to 16 days. This was followed by a decrease in antibody titer.

Although the development of CF antibodies occurred in all cattle infected with Indiana-VS virus at approximately the same rate as in the New Jersey-type VS convalescent cows, the duration of CF response was definitely shorter. In all cows infected with Indiana-VS virus, the CF antibodies disappeared rapidly from circulation and at 50

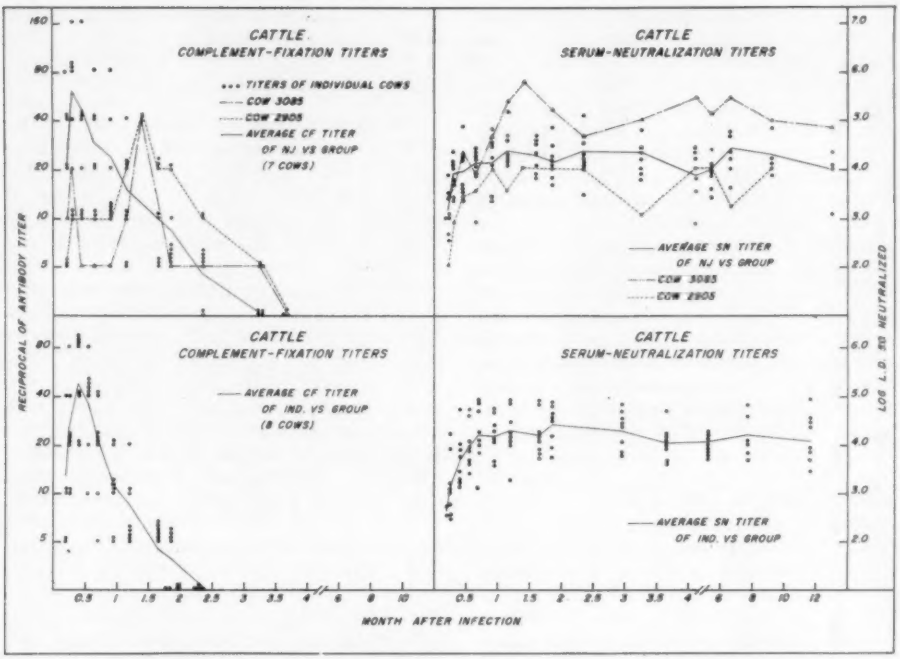

Fig. 1—Complement-fixing and serum-neutralizing antibody titers in cattle serum following experimental infection with New Jersey or Indiana types of vesicular stomatitis virus.

to 75 days postinoculation the serum titers were negative.

Serum-Neutralizing Antibody.—In determining the neutralizing antibody pattern, the same set of New Jersey- and Indiana-vs serum samples used in the CF tests were tested in neutralization tests with dilutions of homologous virus. The average SN titers obtained in these tests are shown (Fig. 1).

In cattle, the variations in the SN antibody levels stimulated by either type of VSV were not appreciably different. All preinoculation serum samples were negative when examined for neutralizing activity. Specific neutralizing antibodies were detected in serum from New Jersey- or Indiana-vs convalescent cattle at 6 to 8 days postinoculation. In the majority of cattle, the neutralizing and CF antibodies appeared at the same time; however, in some cows the CF activity was detected 2 days before any neutralizing antibodies could be demonstrated. During the early convalescent period, frequent changes in the serum antibody levels were observed. The extent and rate of such changes varied in individual animals of both VS groups. In general, the SN antibodies, after attaining high levels within 4 to 5 weeks, remained high but fluctuated in a range of less than 2 logs for the entire test period. The neutralizing index of tested serum samples ranged from 3.8 to 5.6 logs of LD_{50} virus neutralized.

ANTIBODIES IN HORSE SERUMS

Complement-Fixing Antibody.—The CF results, using convalescent serum from 4 horses infected with New Jersey-type VSV and 3 horses infected with Indiana-type VSV, are summarized (Fig. 2).

Horses of both groups responded to infection with CF titers that were higher and persisted longer than those obtained for cattle. Complement-fixing antibodies in the New Jersey-type VS horse serum were observed on the 7th or 8th day, and the maximal titers occurred within the 2nd week postinoculation. During the 3rd week a general decrease in CF antibody titers was found in all animals, although the rate and

magnitude of the declines was variable. In 2 horses, the circulating antibodies became negative in about 72 days postinoculation, whereas in the 2 remaining animals they continued to be detectable at 1:5 dilution for about 30 days longer.

In the horses infected with Indiana-vs virus, the antibody patterns were similar to those found for the New Jersey-vs convalescent horse serum. In the descending phase of antibody levels, however, individual horses differed slightly in quantity and persistence of detectable antibodies. The CF titers of 2 horses, after attaining the highest levels at 12 days, diminished rapidly and disappeared in about 60 days postinoculation, whereas the titer of the 3rd horse decreased slowly and became negative after 4 months.

Serum-Neutralizing Antibody.—The SN activity in convalescent horse serum of both New Jersey- or Indiana-vs groups (Fig. 2) was demonstrated 7 to 8 days postinoculation. During the 3rd and 4th weeks, marked increases were found for the levels of neutralizing antibody, and the indexes for most horses ranged from 4.0 to 5.8 logs of LD_{50} virus neutralized. Neutralizing indexes below 4.0 logs occurred with only 2 convalescent horse serum samples. The circulating antibodies remained at a fairly constant level for a short time and then fluctuated in the range of less than 2 logs throughout the entire test period.

PERSISTENCE OF SERUM-NEUTRALIZING ANTIBODY

In following the persistence of SN antibodies over a long period, it was noticed that, in blood samples obtained irregularly from 5 cows and 1 horse, these neutralizing antibodies were maintained at high levels without significant changes for 19 to 48 months following inoculation (Table 1). Decreases in neutralizing indexes that occurred in some animals were not more than 1 log dilution over the entire test period and can be regarded as change of antibody levels within normal limits.

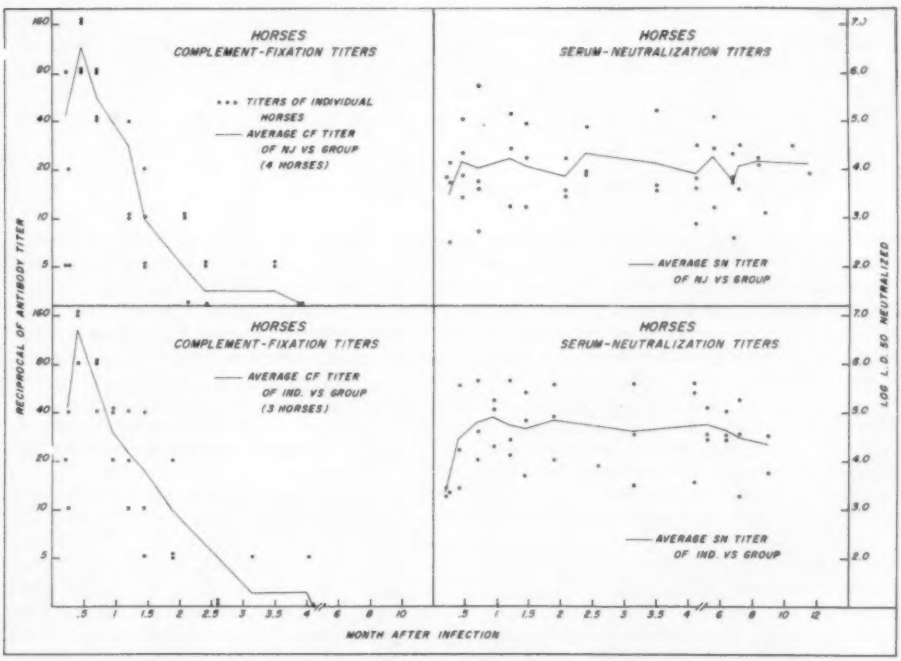
HORSES
COMPLEMENT-FIXATION TITERS
... TITERS OF INDIVIDUAL HORSES
— AVERAGE CF TITER OF NJ VS GROUP (4 HORSES)
HORSES
SERUM-NEUTRALIZATION TITERS
— AVERAGE SN TITER OF NJ VS GROUP
HORSES
COMPLEMENT-FIXATION TITERS
— AVERAGE CF TITER OF IND. VS GROUP (3 HORSES)
HORSES
SERUM-NEUTRALIZATION TITERS
— AVERAGE SN TITER OF IND. VS GROUP
RECIPROCAL OF ANTIBODY TITER
LOG L.D. 50 NEUTRALIZED
MONTH AFTER INFECTION

Fig. 2—Complement-fixing and serum-neutralizing antibody titers in horse serum following experimental infection with New Jersey or Indiana types of vesicular stomatitis virus.

REINOCULATION WITH HOMOLOGOUS VIRUS

The antibody response to re-exposure with holomogous-type virulent virus and the susceptibility to reinfection were determined in a group of recovered animals (1 cow and 4 horses). Two animals out of 5 were immune to re-exposure. Two horses convalescing from Indiana-vs were re-inoculated IDL with 4 ml. of Indiana unfiltered 10% virus suspension of 9.5 and 11.0 months, respectively, after primary infection. They were refractory to the challenge inoculation. Guinea pigs inoculated with the same virus suspension developed typical vs lesions. A cow and 2 horses from a group infected with New Jersey-type virus were re-exposed IDL with 3 ml. of 5% filtered suspension of homologous virus at 7.0, 8.5, and 12.0 months, respectively; they became infected and developed vesicular lesions at the inoculation site. The CF antibodies appeared again in serum of these 5 animals at diagnostic levels and the titer of neutralizing antibodies increased markedly. The CF titers of the samples collected within 8 to 15 days after re-exposure varied from 1:10 through 1:40. An increase of antibody CF titer in serum samples on subsequent days was not observed. In 2 animals, CF titers after reaching a maximum remained unchanged for 2 to 3 weeks, while in a 3rd animal the titer diminished rapidly and decreased to negative in a short time. The SN antibodies rose rapidly following challenge inoculation, reaching approximately a 1-log increase in titer for all animals. After attaining a peak, the SN antibodies for most animals remained unchanged; however, in one horse recovered from Indiana-vs infection but resistant to re-exposure, a small rise in titer lasted approximately 4 weeks and subsequently declined to previous levels.

Discussion

The results of this study confirm and add to the findings and impressions gained [6,7] in the past from observations and testing of natural-occurring and experimental infections of horses and cattle. Serum samples tested for antibody by the direct CF method had specific and reproducible titers. The diagnostic CF titers appeared 6 to 8 days postinoculation. After reaching a peak within 2 weeks, the CF titers gradually declined and disappeared at 110 days or earlier following inoculation. Insignificant cross reactions between immune serum and antigens of the 2 vs types were observed. These occurred mostly with the serum samples of both animal species at 10 to 16 days postinoculation. Serum-neutralizing titers were demonstrated at 6 to 8 days following inoculation. Increased to relatively high levels within 5 weeks, they remained high but fluctuated irregularly for the duration of the test. In general, the immunologic response for most animals followed much the same pattern; however, variations in some individuals were observed.

The CF findings with serum from cows 3085 and 2905 illustrate variations in responses that might sometimes be encountered in individual vs-infected animals. Delayed appearance of vesicles and viremia, which occurred in these 2 animals, could be regarded as factors that caused delayed rise in titer.

The rapid disappearance of CF antibodies in the Indiana-vs convalescent animals might be related to the strain of Indiana VSV. The strain employed in this study was maintained for many years by serial passages in guinea pigs, cattle, and horses. The passage of the VSV in susceptible animals of various species affects the virus characteristics to such an extent that marked changes are usually observed in such properties as

TABLE 1—Serum-Neutralizing Titers of 6 Animals from Which Blood Samples Were Obtained Irregularly After Experimental Infection

Animal	Type of VSV used	Serum titers at month*								
		1.0	1.5	7.0	19.0	21.0	23.0	27.0	36.0	48.0
Cow 3688	New Jersey	4.56	.....	.....	.....	4.39	.....	4.39	.....	.....
Cow 3240	New Jersey	5.70	5.00	4.75	.....	.....	.....	.....	.....	4.74
Cow 3693	New Jersey	4.24	.....	.....	.....	.....	4.30	.....	.....	.....
Cow 3669	Indiana	5.68	.....	.....	.....	.....	.....	5.31	.....	.....
Cow 3702	Indiana	.....	4.18	.....	4.56	.....	.....	.....	.....	.....
Horse 1303	Indiana	5.37	.....	.....	.....	.....	.....	.....	4.52	.....

* Titers expressed as the logarithm of the LD_{50} virus neutralized.

virulence, antigenicity, and species specificity. On the other hand, the early disappearance of antibodies may indicate the existence of antigenic differences between the New Jersey and Indiana types of VSV, or it may be attributed to individual response of animals used in this group.

Serum-neutralizing antibodies fluctuated irregularly at high levels in the blood of recovered animals and maintained this level over a protracted period without significant change in titers. Although no definite conclusions could be reached as to the significance of such fluctuations, they are highly interesting.

In comparing antibody patterns as determined by the CF and SN tests, the study showed that the CF antibodies appeared earlier and increased at higher rates in most cattle and horse serum samples than the observable values of detectable SN antibodies. Furthermore, some samples with high CF activity had similarly high neutralizing titers but, in some instances, samples with low CF titers were associated with high neutralizing titers. The most striking feature was the early disappearance of CF antibodies from circulation at 110 days postexposure as compared with the persistence of neutralizing antibodies at relatively high levels for a protracted period.

The immunity of the recovered animals as measured by virus challenge inoculation was of a short duration. Only 2 horses were resistant when re-exposed to virus of homologous type at 9.5 and 11.0 months after primary inoculation. Individual behavior of these animals or the strain of virus used could be responsible for the outcome of the challenge.

The data obtained on serum collected from re-exposed animals showed that the presence of SN antibodies at high levels does not necessarily indicate active immunity to virus reinoculation.

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SUMMARIO IN INTERLINGUA

Stomatitis Vesicular—Le Comportamento de Anticorpore a Fixation de Complemento e de Anticorpore Sero-Neutralisante in le Sero de Convalescente Bestial e Cavallos

Le comportamento de anticorpore a fixation de complemento e de anticorpore sero-neutralisante esseva determinate in specimens de sero serialmente colligite ab 22 pecias de bestial e ab 8 cavallos que habeva essite inficite experimentalmente con virus de stomatitis vesicular typos New Jersey e Indiana.

Seros testate pro anticorpore per directe fixation de complemento habeva specific e reproducibile titros. Tanto in bestial como etiam in le cavallos, le titros diagnostic appareva 6 a 8 dies post le inoculation e attingeva lor maximo in 9 a 16 dies. Postea, le titros declinava gradualmente e dispareva in 50 a 110 dies.

Titros sero-neutralisante esseva demonstrate 6 a 8 dies post le inoculation. Illos montava a relativemente alte nivellos intra 4 a 5 septimanas. Le titros del anticorpore neutralisante remaneva alte sed fluctuava irregularmente in un campo de variation de minus que 2 log durante le resto del periodo experimental.

Le majoritate del restablite animales esseva susceptibile pro inoculationes provocatori con un virus de typo homologe in que le titro de fixation de complemento habeva disparite ben que le titro sero-neutralisante remaneva alte. Tanto in le animales susceptibile como etiam in le immunes, le titros montava rapidemente post le provocation.

Intravenous Ether Anesthesia in Equine Animals

Albert A. Gabel, D.V.M., M.S.

SUMMARY

Fifty-two horses and mules, including 7 which were clinically ill, were anesthetized with intravenous ether, usually following administration of other drugs. Before and after anesthesia, thorough physical examinations were done and, in many cases, coagulation time, bleeding time, and plasma hemoglobin were determined. Vital signs were recorded at 10-minute intervals during anesthesia. Necropsy and limited histopathologic studies were done on all of the animals, except those surviving clinical illness.

Twenty-four of the 45 experimental animals were assigned at random to 4 groups of 6 animals each and were given choloral hydrate and ether; promazine and ether; choral hydrate, thiopental, and ether; or promazine, thiopental, and ether. Seven liters of 5% ether in 0.9% sodium chloride solution was injected at a rate which kept the animals in a light plane of surgical anesthesia. Blood pressure and electrocardiographic recordings were made continuously during the induction and maintenance of anesthesia of these animals.

Results from the 52 animals showed that promazine and chloral hydrate were equally effective and satisfactory as preanesthetic agents. When anesthesia was induced with intravenous ether, a long period of moderate struggling occurred, but this did not occur when thiopental was used for induction following injection of one of the premedicaments mentioned. Good anesthesia was maintained with intravenous ether. The depth of anesthesia could be fairly easily controlled by a person familiar with the signs of anesthesia. The most important advantage of the method was that recovery was completely free of struggling but the recovery period was rather long. Following an average of 74 minutes of surgical anesthesia, the animals stood after an average of 109 additional minutes. Little evidence was found of serious functional or morphologic change due to the intravenous ether.

One animal died as a result of being taken to stage IV anesthesia inadvertently by the too rapid injection of ether-saline solution. One animal died while in a light plane of anesthesia. Orthopedic surgery was being done at the time of death. There is some evidence that fat emboli may have caused the death of this animal. One animal died due to laryngospasm, probably caused by injury to the larynx prior to anesthesia. Two animals developed azoturia, probably due to struggling in the casting harness while the carotid artery was being isolated (for the recording of blood pressure) prior to induction of anesthesia.

The margin of safety with intravenous ether appears to be rather narrow. This anesthetic method appears to be less safe than others which are available. Another disadvantage of the method is that considerable time is needed to prepare the large quantity of ether-sodium chloride solution required.

A useful application of the method may be to extend, for a short period, anesthesia induced by other drugs when recovery must be free of struggling.

Received for publication Sept. 1, 1960.

From the Department of Veterinary Surgery and Radiology, College of Veterinary Medicine, Ohio State University, Columbus.

This article is based on part of a thesis submitted in partial fulfillment of requirements for the M.S. degree.

This project was supported by the Ohio State University Development Fund.

The author wishes to acknowledge the advice and encouragement of Dr. J. Jacoby, formerly chairman of the Department of Anesthesia, Ohio State University (now chairman, Department of Anesthesia, Marquette University); Dr. Richard L. Rudy, chairman of the Department of Veterinary Surgery and Radiology, Ohio State University; and Dr. C. R. Smith, chairman of the Department of Veterinary Physiology and Pharmacology, Ohio State University.

INTRODUCTION

There is a great need for a method of general anesthesia in horses, in which induction and recovery are rapid and free from struggling. The anesthesia should also be safe, easily administered, easily controlled, and dependable.

The purpose of this article is to report and evaluate the effects of intravenous diethyl ether anesthesia following one of several drug combinations in 52 horses and mules and to evaluate the method.

Review of Literature

Burkhardt[5] determined that 5% ether in 0.9% sodium chloride solution was safe for anesthesia of laboratory animals. He also found that a 7% solution produced a slight hemoglobinuria. He was the first to use intravenous ether anesthesia successfully in man. The method has not come into general use in man, despite the satisfactory results recorded by many authors. Doggliotti,[7] in 1939, stated that, "the method is inadvisable" because of the difficulty of regulating the depth of anesthesia and because of the large volume of fluid required.[4, 14] Adams,[1] in 1944, thoroughly reviewed the literature on intravenous ether.

The use of intravenous ether in horses was described and strongly recommended by Longley in 1950.[10] He used the method to maintain anesthesia after induction with a "quick injection" of thiopental sodium in 5 animals. In a 6th, he induced and maintained anesthesia with ether. Guard[13] also has successfully used intravenous ether following a "quick injection" of thiopental sodium.

Conner[6] reported the use of intravenous ether anesthesia of 14 cattle in 1951. In each case, the animal was cast prior to anesthesia, and in all except one case, induction was by rapid intravenous injection of pentobarbital sodium. Only 1 of the animals was allowed to recover; the rest were euthanatized and immediately embalmed for anatomy dissection. Two calves, each under 3 months of age, died during anesthesia.

Jacobsen,[16] in 1955, reported the satisfactory use of intravenous ether anesthesia in 4 gilts during caesarian sections. Thiopental sodium was used for induction of anesthesia.

Materials and Methods

Ten of the experimental animals were horses from the clinic and were anesthetized prior to euthanasia. Thirty-five of the experimental animals were the least expensive horses and mules which could be purchased in the Columbus, Ohio, area. Their average age (estimated by examination of teeth) was 16 years, and their average estimated weight was 920 lb. None of them had signs of infectious disease, but several were thin. Six of these animals were in each of 4 drug-combination groups: chloral hydrate and ether; promazine* and

* Sparine, supplied by Wyeth Laboratories, Philadelphia, Pa.

Fig. 1—Method of restraint.

ether; chloral hydrate, thiopental sodium** induction, and ether; and promazine, thiopental sodium induction, and ether. Animals were assigned to groups at random. One mule was included in each group, except the last group mentioned.

In addition to the experimental animals, 7 with nearly hopeless clinical cases were anesthetized for surgery.

Before anesthesia, a thorough physical examination and urinalysis were done and, in many cases, Lee White coagulation time,[12] bleeding time, and plasma hemoglobin[13] were determined.

A preanesthetic dose of 0.5 mg. of promazine per pound or 0.3 ml. of 7% chloral hydrate per pound of body weight was administered intravenously. The animals were cast a few minutes after injection of chloral hydrate or promazine. Induction with thiopental sodium, in the two groups, was accomplished by successive injections of 0.167 Gm. at 30- to 60-sec. intervals until spinal reflexes were obtundent. An average of 1.57 Gm. of thiopental was required to induce anesthesia.

Blood pressure was recorded in most of the 35 experimental animals (Fig. 1). Procaine was injected in the skin and muscle dorsal to the incision site used for isolation of the carotid artery. This site was halfway between the point of the shoulder and the head, just dorsal to the jugular vein. Twelve inches of an 18-inch flexible aluminum alloy cannula (inside diameter 3/16 inch) was introduced inside the carotid artery toward the heart and ligated. The incision in the carotid artery was sutured when the recording was finished. The cannula was connected to a Statham P23A strain gauge which had been calibrated with a mercury manometer. The transducer was placed at heart level. Blood pressure and electrocardiographic recordings were made continuously during induction and maintenance of anesthesia on a direct-writing Sanborn "Twin Viso" recorder. Other vital signs and signs of anesthesia were recorded at 10-minute intervals.

Thiopental sodium and ether were injected through polyethylene tubing* which was put into the jugular vein with a special thin-walled 10-gauge needle. A Fisher clamp was used to control the continuous flow of ether-sodium chloride solution. Thirty-five experimental animals were maintained in light surgical anesthesia (plane 1 or 2) until 7 liters of 5% ether in 0.9% sodium chloride had been injected.

When ether injection was completed, Lee White coagulation time, bleeding time, and plasma hemoglobin were again determined. Other clinical laboratory studies, reported in "Results," were also done on some animals.

Following anesthesia, animals were observed daily. On the 2nd day after anesthesia, a thorough physical examination and urinalysis were again done. In most cases, the horses were used for student surgical practice (chloral hydrate anesthesia) 2 to 5 days after anesthesia.

Necropsy performed immediately after death included removal and gross examination of the heart,

** Pentothal, supplied by Abbott Laboratories, North Chicago, Ill.

* PE 260, Clay Adams, New York, N.Y.

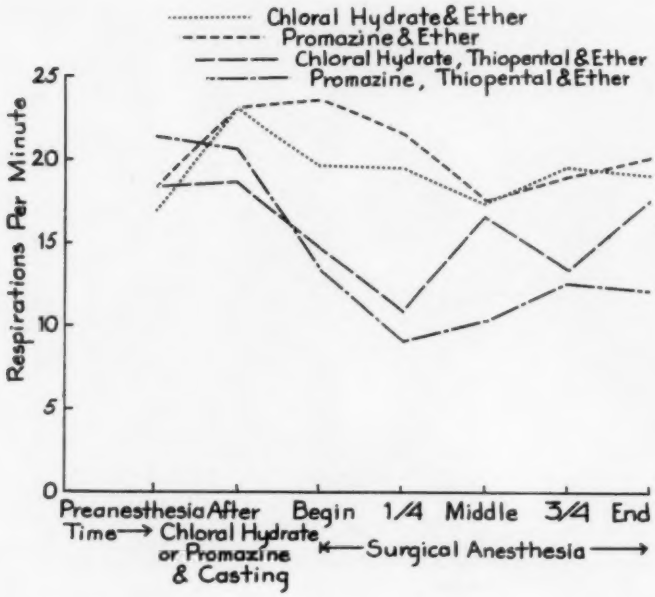

Fig. 2—Mean respiratory rates. Result of analyses of variances: The groups of animals in which thiopental was used had significantly slower mean respiratory rates than the animals in which no thiopental was used, at the beginning, after one-fourth,** and after three-fourths* of the periods of surgical anesthesia.**

* Significant at 95% confidence level. ** Significant at 99% confidence level.

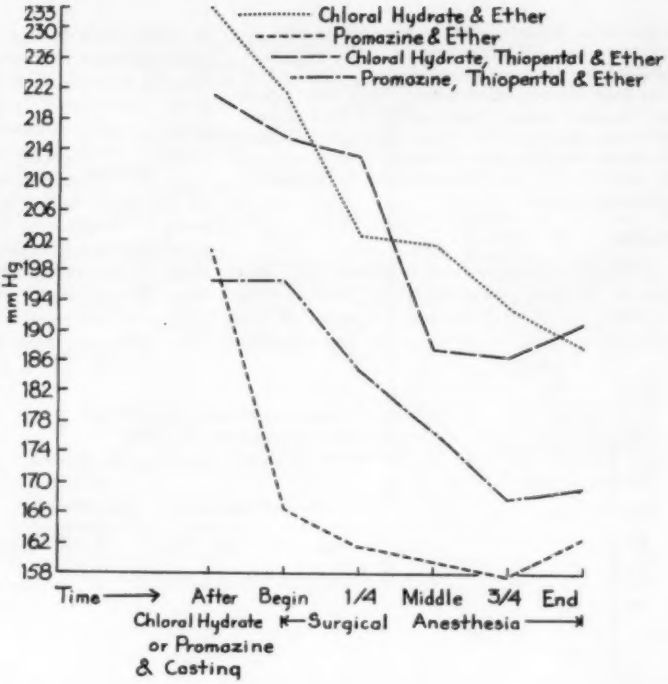

Fig. 3—Mean systolic blood pressures. Result of analyses of variance: The groups of animals in which promazine was used had significantly lower mean systolic blood pressures than the animals in which chloral hydrate was used, after casting,* at the beginning,** after one-fourth,** at the middle,* after three-fourths,* and at the end* of the periods of surgical anesthesia.

* Significant at 95% confidence level. ** Significant at 99% confidence level.

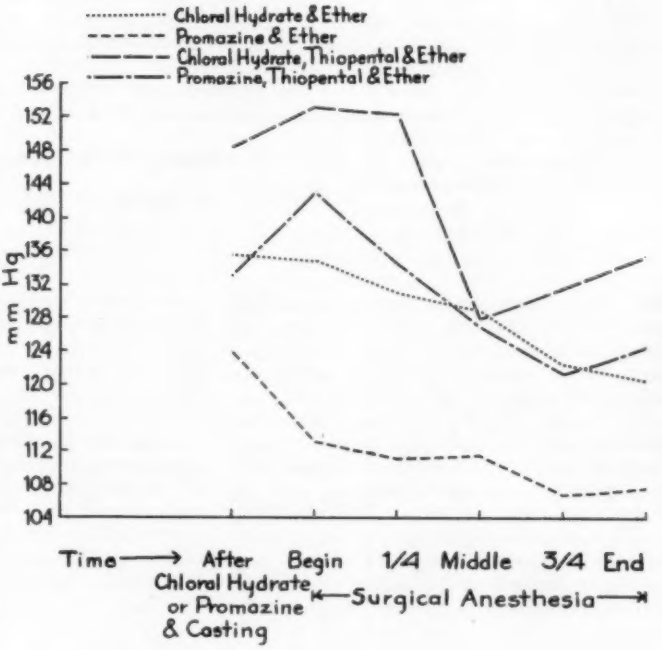

Fig. 4—Mean diastolic blood pressures. Result of analyses of variance: The groups of animals in which promazine was used had significantly lower mean diastolic blood pressures than groups of the animals in which choral hydrate was used after one-fourth* of the period of surgical anesthesia. The groups of animals in which thiopental was used had significantly higher mean diastolic blood pressures than the groups of animals in which no thiopental was used, at the beginning,** after one-fourth,* and at the end* of the periods of surgical anesthesia.

* Significant at 95% confidence level. ** Significant at 99% confidence level.

lungs, liver, kidneys, and, in some cases, the adrenals, brain, and other organs. Histopathologic studies using the hematoxylin-eosin staining method were done on sections of kidneys from each animal and on other organs from animals in which complications occurred. The brain, lungs, and kidneys of several animals were examined for evidence of fat emboli using the frozen section technique and oil-red-O fat stain.

Results

Clinical Observations.—Promazine and chloral hydrate were equally effective and satisfactory as preanesthetic agents. When anesthesia was induced with intravenous ether, a long period of moderate struggling occurred, but this did not occur when thiopental was used for induction. Satisfactory, fairly easily controlled anesthesia was produced by intravenous ether.

Nystagmus usually started during induction and continued during an average of three fourths of each period of light surgical anesthesia (plane 1 and 2), and the early part of recovery. During these periods of anesthesia, pupils were constricted or less than one half dilated and pupillary reflexes were absent; anal and corneal reflexes were present, and usually a weak palpebral reflex could be elicited. Spontaneous movements occurred in 27 of the 52 animals during the periods of surgical anesthesia, e.g., while the animals would not respond to painful stimuli applied to the legs, abdomen, or neck.

In the 35 experimental animals, 7 liters of 5% ether in 0.9% sodium chloride maintained anesthesia for an average of 74 minutes; the average time from the end of ether injection to return of spinal reflexes was 26 minutes; and the animals stood after an average of 109 additional minutes. During recovery, no struggling occurred, and animals seldom attempted to rise before they were able to do so safely. Excessive salivary and bronchial secretions did not occur, although anticholinergic drugs were not used.

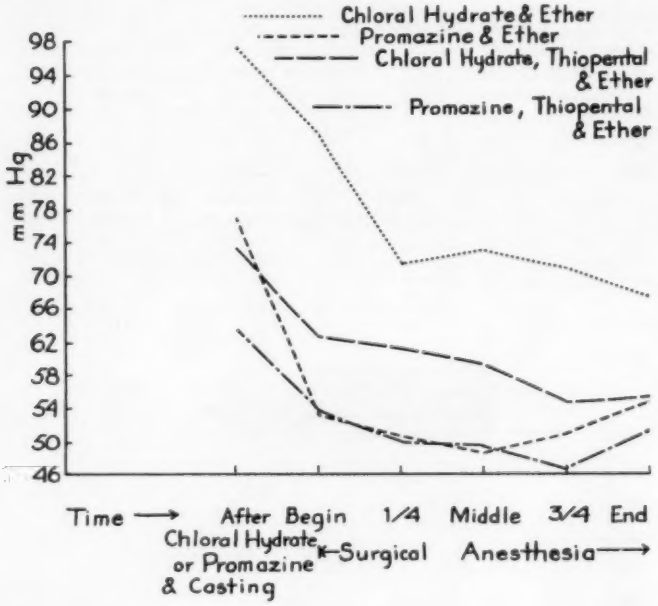

Fig. 5—Mean pulse pressures. Result of analyses of variance: The groups of animals in which promazine was used had significantly lower mean pulse pressures than the animals in which choral hydrate was used, at the beginning, after one-fourth,** at the middle,** and after three-fourths* of the periods of surgical anesthesia.**

* Significant at 95% confidence level. ** Significant at 99% confidence level.

Carotid isolation and introduction of a cannula for recording blood pressure in the experimental animals resulted in occlusion of the artery in only one fourth of the animals.

Physiologic Changes.—The mean values of the vital signs for each drug-combination group have been plotted (Fig. 2–6). The factorial method of analysis of vari-

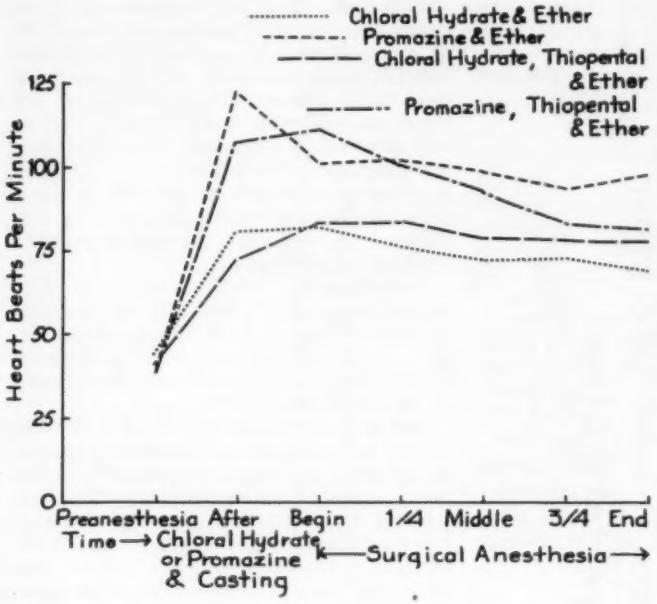

Fig. 6—Mean pulse rates. Result of analyses of variance: The groups of animals in which promazine was used had significantly faster mean pulse rates than the animals in which chloral hydrate was used, after casting, at the beginning,** after one-fourth,** and at the middle* of the perods of surgical anesthesia.**

* Significant at 95% confidence level. ** Significant at 99% confidence level.

ance was used, except on the data obtained prior to anesthesia, since only one factor (chloral hydrate-promazine) was involved. Below each figure the statistically significant differences in mean values of vital signs from the drug combinations are recorded.

In 3 cases, one of the following cardiac arrhythmias was found during anesthesia: ventricular premature extrasystole, auricular premature systole, and sinus arrhythmia.

Average change in rectal temperature was negligible, but individual increases or decreases of up to 2.8 F. occurred.

In 25 animals, coagulation time averaged 14.8 minutes before anesthesia and 10.5 minutes when ether injection stopped; this was an average decrease of 29%. By analysis of variance, the difference was found to be significant.* There was no significant difference found in the decrease in coagulation time between the drug-combination groups.

In 29 animals the bleeding time averaged 16.4 minutes before anesthesia and 14.6 minutes when ether injection stopped. This difference was not found to be significant. There was no significant difference in the decrease found in average bleeding time between the drug-combination groups.

The plasma hemoglobin in 17 animals averaged 0.96 mg./100 cc. before anesthesia, 9.62 mg./100 cc. when ether injection stopped, 8.53 mg./100 cc. one half hour after ether injection stopped, and 7.89 mg./100 cc. 2 hours after ether injection stopped. The highest level found was 24.5 mg./100 cc. Detailed data and discussion of the effects of the intravenous ether anesthesia on the blood of these horses is reported elsewhere.[11]

Hemoglobinuria did not occur. Glycosuria occurred in one fourth of the 45 samples tested after ether injection was stopped. Specific gravity of the urine did not change significantly, except in animals anesthetized for long periods (in these it decreased). Proteinuria did not occur and there were no other significant changes in the urine, except in those animals which developed azoturia. Limited data showed that plasma and blood volume decreased and that hematocrit level increased. Serum sodium and chloride increased in the few individuals in which these determinations were done. Large quantities of ether-sodium chloride solution had been injected in these cases.

* Significant at 95% confidence level.

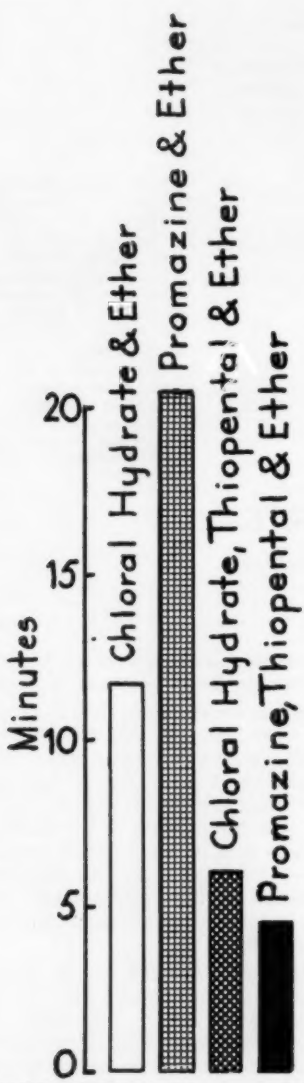

Fig. 7—Mean induction times. Result of analysis of variance: The group of animals given promazine and ether had a significantly longer mean induction time than those given chloral hydrate and ether. The group of animals in which thiopental was used had significantly** shorter mean induction times than the animals in which no thiopental was used. Significant** interaction appears to have occurred between the drugs used, e.g., the mean induction time for the promazine and ether group was longer than would be predicted from the mean induction times of the other 3 groups.**

* Significant at 95% confidence level. ** Significant at 99% confidence level.

Pathologic Changes.—Intravenous ether anesthesia caused albuminous degeneration of the kidneys and necrosis of occasional tubular cells, especially in the proximal convoluted tubules. Slight edema of the brain, especially the cerebral motor cortex, was observed in 4 animals which died during anesthesia. Marked edema of the brain occurred in 2 animals in which large quantities of ether-sodium chloride solution was injected to maintain surgical anesthesia until death occurred.

Complications and Deaths.—A 375-pound foal (clinical case) died as a result of being taken to stage IV anesthesia inadvertently after 95 minutes of anesthesia. Preanesthetic 7% chloral hydrate had been given intravenously (250 ml.).

One animal died suddenly after 98 minutes of anesthesia, while in a light plane of anesthesia. Moderately traumatic orthopedic surgery (sliding bone graft to fuse 1st and 2nd phalanges) was done during the anesthesia. Fifteen grams of chloral hydrate, 4 Gm. of thiopental sodium, and 15.5 liters of ether-sodium chloride solution had been injected to induce and maintain anesthesia. Microscopic lesions which appeared to be fat emboli, demonstrated in the lungs and cerebellum, may have caused death. No comparable lesions could be demonstrated in 14 of the other animals anesthetized, nor in 8 controls slaughtered by rifle fire.

Two animals (experimental cases) were unable to rise following anesthesia, due to azoturia. One of these animals died 10 hours after anesthesia. In each of these animals, blood nonprotein nitrogen (NPN) and urinary creatine and creatinine increased; urine pH decreased to 5; hyaline degeneration of skeletal muscle was found microscopically; and the kidneys had the microscopic changes described in azoturia.[19] These changes were not found in horses which did not have signs of azoturia following ether anesthesia.

Another animal (experimental case) was unable to rise after anesthesia. Weakness before anesthesia was probably important in contributing to this. No evidence of azoturia was found in clinical laboratory studies.

One animal (experimental case) died of laryngospasm, probably caused by injury to the larynx prior to anesthesia.

Euthanasia, performed on a healthy 325-lb. foal which had a severe leg injury, was

by rapid injection of ether-sodium chloride solution. After injection of 2 liters of solution during 8 minutes, spinal reflexes were still present. One minute later, respiration stopped and, after 40 seconds more, heart action stopped.

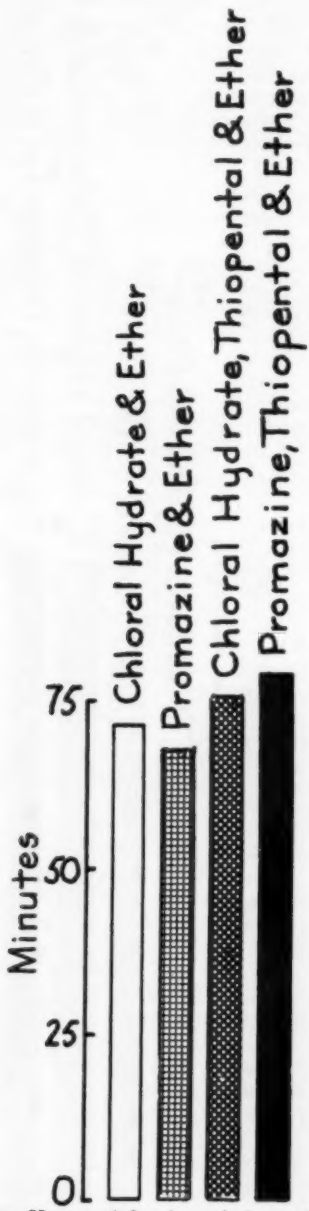

Fig. 8—Mean periods of surgical anesthesia.

Discussion

The spontaneous movements which occurred during surgical anesthesia (e.g., while the animals would not respond to painful stimuli applied to the legs, abdomen, or neck) was probably due to stimulation of unknown cause of motor centers or motor pathways. Similar observations have been made on dogs during recovery from pentobarbital sodium anesthesia and on horses during light choral hydrate anesthesia and thiopental sodium anesthesia. Perhaps the spontaneous movements are similar to a phenomena described by Adriani [2] in dogs anesthetized with vinyl ether.

Thiopental depressed the respiratory rate, significantly,** as expected (Fig. 2).[8,20]

Systolic blood pressure was significantly* lower after the administration of promazine and casting than after the administration of chloral hydrate and casting (Fig. 3). Late in anesthesia, the difference between the 2 groups became less marked, probably due to the promazine and choral hydrate being inactivated and the high concentration of ether over a long period masking the effect of these drugs.

Diastolic blood pressure was slightly lower* during the early part of anesthesia in the groups which were given promazine (Fig. 4). On the average, the animals in which anesthesia was induced with thiopental had significantly** higher diastolic blood pressure, during the early part of anesthesia, than did those in which anesthesia was induced with ether. Mean blood pressure was also significantly* higher during this period in animals in these groups. Slow injection of thiobarbiturates in dogs [8,20] and monkeys [8] sometimes causes an increase in blood pressure which is accompanied by a decrease in the volume of the spleen, kidneys, and extremities. It appears that a similar response occuurs in horses.

The pulse pressure was significantly** lower during most of anesthesia in the animals given promazine (Fig. 5). This occurred because systolic blood pressure was lower and diastolic blood pressure was much less lower, especially early in anesthesia, in

* Significant at 95% confidence level.
** Significant at 99% confidence level.

Fig. 9—Mean time from "end of ether injection to return of spinal reflexes." Analyses of variance: The group of animals in which promazine was used had significantly** longer mean periods "from end of surgical anesthesia to return of spinal reflexes" than the groups of animals in which chloral hydrate was used. Significant* interaction appears to have occurred between the drugs, e.g., the animals given chloral hydrate, thiopental, and ether had a shorter mean period "from end of ether injection to return of spinal reflexes" than would be predicted from the effect of these drugs in animals of the other groups.

* Significant at 95% confidence level. ** Significant at 99% confidence level.

Fig. 10—Mean times "from return of spinal reflexes to standing."

animals given promazine than in animals given chloral hydrate.

The pulse rate after administration of promazine and casting was significantly** higher than after administration of chloral hydrate and casting (Fig. 6). This result is in accord with Marey's law,[3,22] due mainly to the aortic and carotid sinus reflexes,[3,10,22] since the systolic and pulse pressures were significantly lower during this period in the groups of animals given promazine.

Induction time was very short when thiopental was used, since it was injected relatively rapidly (Fig. 7). It took nearly twice as long to induce anesthesia with ether when promazine had been given, as when chloral hydrate had been given as the preanesthetic agent. This result was probably due to the greater cerebral depressant effect of chloral hydrate,[17] or perhaps the doses of the 2 drugs were not comparable. The indicated significant** interaction is difficult to explain. The additional variable of rate of thiopental injection may have caused it.

The length of anesthesia in animals of the 4 groups was not significantly different (Fig. 8), although the animals given thiopental had slightly longer periods of anesthesia, perhaps because they were given more total anesthetic agents.

The time from the end of ether injection to return of spinal reflexes was shorter when chloral hydrate was used than when promazine was used as the preanesthetic agent (Fig. 9). This is expected, since the effect of promazine lasts longer than the effect of chloral hydrate. The indicated significant interaction is probably not a true interaction, since the period from return of spinal reflexes to standing (Fig. 10) was nearly as long or longer in the groups of animals given chloral hydrate and thiopental than it was in animals of the other 3 groups.

The time from return of spinal reflex to standing was not significantly different between the 4 groups (Fig. 10), but the animals given thiopental required slightly more time since they were given more total anesthetic agents. Time required for recovery from intravenous ether anesthesia appears to be longer than recovery time after inhalation ether anesthesia.* This difference is difficult to explain.

The degree of hemolysis found should have little harmful effect.** Hemolysis by ether "may be due either to a decreased resistance of the cell envelope or to a change in the state of its colloids."[21]

The azoturia which occurred in 2 horses was probably not caused by ether anesthesia, but was probably caused by the restraint in the casting harness.[19] Each of these horses struggled during the 45 minutes required to cast the animal, to isolate the carotid, and start recording data prior to induction of anesthesia.

It appears that the margin of safety during intravenous ether anesthesia is narrow, especially after large quantities have been injected. Since ether is very soluble in body fluids and fats,[9] they act as a buffer system early in ether anesthesia. However, after a long period of ether anesthesia, the concentration in body fluid and fat becomes nearly equal to that of blood, and ether level of blood (and to the brain) rises more sharply as a result of a given injection. The great solubility of ether in body fluid and fat also cause the relatively long induction and recovery periods.

* Significant at 95% confidence level.
** Significant at 99% confidence level.

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SUMMARIO IN INTERLINGUA

Anesthesia per Ethere Intravenose in Animales Equin

Cinquanta-duo cavallos e mulos, incluse 7 que esseva clinicamente malade, esseva anesthesiate con ethere intravenose—usualmente post le administration de altere drogas. Ante e post le anesthesia, meticulose examines physic esseva effectuate, e—in multe casos—le tempore de coagulation, le tempore de sanguination, e le hemoglobina del plasma esseva determinate. Signos vital esseva registrate a intervallos de 10 minutas durante le anesthesia. Necropsias e limitate studios histopathologic esseva executate pro omne le animales que non superviveva a lor morbo clinic.

Vinti-quatro del 45 animales experimental esseva distribuite aleatorimente in 4 gruppos de 6 animales cata un e tractate con (1) hydrato de chloral e ethere, (2) promazina e ethere, (3) hydrato de chloral, thiopental, e ethere, o (4) promazina, thiopental, e ethere. Septe litros de 5% de ethere in un solution de 0, 9% de chloruro de natrium esseva injicite a un rapiditate que teneva le animales a un leve nivello de anesthesia chirurgic. Registrationes del tension de sanguine e electrocardiogrammas esseva executate continuemente durante le induction e le mantenentia de anesthesia in iste animales.

Le resultatos del observationes in omne le 52 animales monstrava que promazina e hydrato de chloral esseva equalmente efficace e satisfactori como agentes preanesthetic. Quando le anesthesia esseva inducite con ethere intravenose, un prolongate periodo de debattimento occurreva, sed isto non occurreva quando thiopental esseva usate in le induction del anesthesia post le injection de un del premedicationes mentionate. Un bon forma de anestheia esseva mantenite con ethere intravenose. Le profundor del anesthesia esseva satis facile a regular per un subjecto familiar con le signos de anesthesia. Le plus importante avantage del methodo esseva que le recuperation esseva completemente libere de debattimento, sed le periodo de recuperation esseva satis longe. Post un intervallo medie de 74 minutas de anesthesia chirurgic, le animales stava in pede post un intervallo additional medie de 109 minutas. Pauc evidentia esseva trovate pro serie alterationes functional o morphologic in consequentia del ethere intravenose.

Propagation of Equine Arteritis Virus in Monolayer Cultures of Equine Kidney

William H. McCollum, Ph.D.; E. R. Doll, M.S., D.V.M.; James C. Wilson, M.S.; Catherine B. Johnson, A.B.

SUMMARY

1) The virus of equine arteritis was recovered from equine tissues and was progagated serially in primary monolayer cultures of equine kidney.

2) The virus produced a cytopathic effect (CPE) which proceeded to complete necrosis of cultures of equine kidney.

3) Horses were infected by intramuscular or intranasal injections of 7th, 10th, 25th, 46th, and 68th kidney cell passages of the Bucyrus strain of virus. Severity of the experimental disease was reduced in succeeding passages of virus in tissue culture. The 16th and 40th passages of the Penn strain of virus infected horses without causing visible signs of disease.

4) All horses exposed to virus propagated in tissue culture developed immunity which resisted challenge to the parent Bucyrus strain of virus 3 weeks later.

5) Horses exposed to virus developed circulating antibodies which inhibited the CPE of the virus in tissue culture.

6) The Bucyrus strain of virus was viable after 6 years of storage at −20 C. in a whole fetal spleen. The 44th kidney cell passage was viable at 75 days when stored in tissue culture medium at 4 C., but survived for only 2 days at 37 C. and for 20 but not 30 minutes at 56 C.

INTRODUCTION

A filterable agent causing arteritis of horses and abortion by mares was isolated and described by Doll *et al.* They found this virus to be transmissible from horse to horse by contact and to be infective when injected intranasally, intratracheally, subcutaneously, or intravenously. The virus was found in the spleens and lungs of aborted fetuses, in the spleens of young and adult horses that died 5 or 6 days after subcutaneous or intravenous inoculation, and in the blood during the febrile period. This virus was not observed to produce evidence of infection in rabbits, hamsters, guinea pigs, mice, or chicken embryos.

Subsequently, various cell cultures were investigated for their capacity to support propagation of the virus. Initial attempts to propagate equine arteritis virus were made with the following cell culture systems: HeLa cells; Hep II cells; Chang's conjunctiva; Henle's intestine; and primary monolayer cultures of chicken embryo kidney, hamster kidney, bovine kidney, and equine kidney. Only in the latter was there evidence of infection as indicated by visible cytopathologic changes in the cultures.

This report describes the propagation of equine arteritis virus in equine kidney cell cultures.

Materials and Methods

Cell Cultures.—Cell cultures were prepared from cortices of kidneys from horses of various ages, according to the trypsin digest method of Youngner.[3] The cells were grown at 37 C. in a medium containing 10.0% horse serum, 0.5% lactalbumin hydrolysate (LAH), and either 89.5% Eagle's medium or 89.5% Earle's solution (ES). In Leighton tubes, 1.2 ml. of 0.5% cell suspension was used; whereas, in 32-oz. prescription bottles, 80.0 ml. was used. The medium was replaced on the 3rd day if cultures were not seeded with virus at this time. Otherwise, when the cultures were ready for use, the nutrient medium was removed, the cell monolayer was washed once or twice with phosphate-buffered saline solution (PBS), and the nutrient medium was replaced with a maintenance medium consisting of 2.0% calf serum, 0.5% LAH, and either 97.5% Eagle's medium or 97.5% ES. Penicillin (100 units/ml.), streptomycin (100 μg./ml.), and nystatin (10 units/ml.) were used in all mediums.

Virus Source.—The spleen of a fetus aborted in March, 1959, due to experimental infection, and

Received for publication Sept. 2, 1960.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington. Published by permission of the director of the station.

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station supported by a grant made by the Grayson Foundation, Inc.

spleens of fetuses aborted in 1953, 1954, and 1955, due to naturally acquired infection, served as sources of the virus. Twenty per cent suspensions of splenic tissues were prepared by mechanically chopping the tissue into fine pieces using PBS as diluent. The supernatant fluid collected after centrifuging at 1,000 relative centrifugal force (r.c.f.) for 5 minutes served as the inoculum for cell cultures.

Isolation of Virus in Tissue Culture.—Cultures of equine kidneys, grown on 11 x 22 cover slips contained in Leighton tubes, were inoculated (0.1 ml. per tube) with supernatant fluids prepared as described. These cultures were examined microscopically each day. The fluids were collected from these cultures after 5 or 6 days, and 0.2-ml. volumes were passed to Leighton tubes containing 10-day-old kidney cell cultures. Cover slips were removed from tubes at various intervals and stained according to the May-Grunwald-Giemsa procedure.[2]

Horse Inoculations.—Horses of various ages, known not to have had prior exposure to arteritis virus were employed. Fluids from the 7th, 10th, 25th, 46th, and 68th passages of the Bucyrus strain and the 16th and 40th passages of the Penn strain of arteritis virus were administered intramuscularly, intravenously, or intranasally. If the horse survived, it and a control horse were inoculated 3 weeks later by intranasal instillation of 10 ml. of supernatant fluid from a 20% suspension of lung tissue from the fetus aborted March, 1959. White blood cell counts were made daily on selected individuals, and rectal temperatures were recorded twice daily.

Estimation of Virus Content.—Tenfold dilutions of culture fluids containing virus were made in maintenance medium. One-milliliter volumes of appropriate dilutions were distributed in Leighton tubes containing suitable cultures of kidney cells. Duplicate tubes were inoculated for each dilution. All tubes were examined microscopically each day for 6 days. For a simple estimate of viral content, the titer was designated as the highest dilution of virus producing a cytopathic effect (CPE).

Results

Isolation and Propagation of Arteritis Virus in Tissue Culture.—The initial passage of the Bucyrus strain (March, 1959, fetus) did not produce cytopathic evidence of infection. The cell cultures of the 2nd passage were incubated for 6 days. Areas of necrosis were observed in both stained and unstained preparations. Virus of the 3rd passage produced rapid and complete necrosis of the kidney cells within 3 days. The effect of the virus on similar cultures has remained constant in more than 80 serial passages.

The cytopathic changes (Fig. 1) were characterized initially by increased optical density and refractivity to light and by rounding of the cells. This was followed quickly by shrinking of the cell, pyknosis, karyorrhexis, and fragmentation. Most affected cells become detached from the glass.

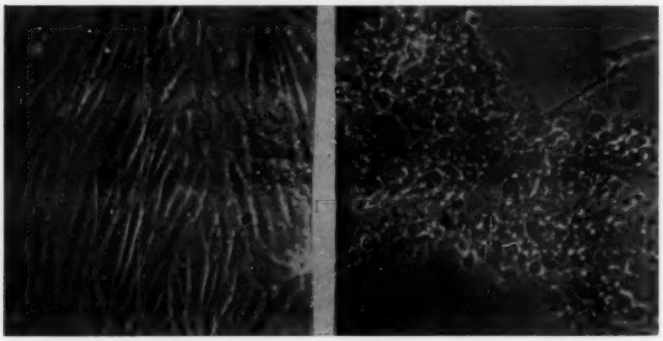

Fig. 1—Cell cultures of equine kidney. Normal cell (left). Cytopathic effect of arteritis virus: nearly all cells are rounded or shrunken (right). Phase-contrast; x 193.

The success of the initial trial for isolation of the arteritis virus suggested the desirability of examining tissues collected from fetuses aborted due to natural occurrences of the disease coming to our attention since 1953. These tissues had been stored at −20 C. since collection. Spleens of fetuses from 3 separate and apparently unrelated epizootics were available. These epizootics occurred in 1953 (Bucyrus strain), 1954, and 1955 (Penn strain). Virus was recovered during the 1953 and 1955 epizootics, but not from tissues collected in 1954. Behavior of these isolates in tissue culture was not observed to differ appreci-

ably from the virus recovered from the fetus of March, 1959.

Concentration of Arteritis Virus at Various Intervals After Inoculation of Tissue Cultures.—Results of a typical titration are recorded (Table 2). The maximum concentration of virus was 10^6 tissue culture infective dose per milliliter and occurred 24 to 36 hours after inoculation of a cell culture contained in a 32-oz. prescription bottle with 3 x 10^5 tissue culture infective doses. There was a gradual, but progressive, drop in titer after 36 hours.

Inoculation of Horses with Virus Propagated in Tissue Culture and Subsequent Challenge with Parent Virus.—These results are summarized (Table 1). The horse exposed to the 7th tissue culture passage of the Bucyrus strain died on the 6th day after inoculation. It had typical signs of the disease, and the gross and microscopic lesions were characteristic of infection by the arteritis virus. A horse inoculated with 10th passage of the Bucyrus strain had a high thermal response (106.0 F.), pronounced leukopenia, and marked clinical signs. It survived, and its immunity and that of a control horse was challenged 3 weeks later with parent virus. The principal horse developed no signs of disease, but the control developed signs typical of infection with arteritis virus. The horse exposed to the 25th tissue culture passage developed a thermal response of 104.8 F. and severe leukopenia, but only moderate signs of disease. Its immunity resisted challenge 3 weeks later, while the control developed severe disease. The 46th tissue culture passage of the Bucyrus strain, when given to a susceptible horse, produced a moderate thermal response (103.1 F.) and slight leukopenia, but no clinical illness. When the immunity of the principal horse and a control was challenged 3 weeks later, the horse previously exposed to virus propagated in tissue culture resisted challenge, whereas the control was infected as evidenced by thermal response of 105.8 F. and by the development of typical signs. The 68th tissue culture passage of the Bucyrus strain produced a mild thermal response (102.8 F.) when given intramuscularly to a susceptible horse, but other signs of the disease were not observed. It resisted challenge by parent virus 3 weeks later, while the control was severely affected.

The 16th and 40th kidney cell passages of the Penn strain were administered to horses. Thermal responses of 103.7 and 104.0 F., respectively, were recorded for these 2 passages. Each horse developed

TABLE 2—Concentration of Arteritis Virus at Various Intervals After Inoculation of Equine Kidney Cell Culture

Hours post-inoculation	t.c.i.d*
Inoculum**	5.0
24	4.0
30	6.0
36	6.0
48	5.0
54	5.0
78	5.0
128	4.0
168	2.0
192	3.0

* Tissue culture infective doses per milliliter, expressed as logarithm to base 10.
** 3.0 ml. into 50 ml. of medium.

TABLE 1—Horse Inoculated with Arteritis Virus Propagated in Tissue Culture; Subsequent Challenge with Parent Virus

Horse	Virus* strain and kidney cell passage	Maximum temp. (F.)	Leukopenia**	Remarks†	Subsequent challenge with parent virus	
					Maximum temp. (F.)	Remarks
739	BS_7	106.3	S	Died 6th day	.....	.....
743	BS_{10}	106.0	S	SS	101.0	NVS
743 Control					106.8	Died 9th day
744	BS_{25}	104.8	S	MS	100.6	NVS
744 Control					105.4	SS
719	BS_{40}	103.1	M	NVS	101.0	NVS
719 Control					105.8	SS
724	BS_{68}	102.8	...	NVS	101.2	NVS
724 Control					106.6	SS
720	PS_{16}	103.7	M	NVS	101.0	NVS
720 Control					105.9	SS
707	PS_{40}	104.0	M	NVS	100.8	NVS
707 Control					106.6	SS

* BS = Bucyrus strain; PS = Penn strain.
** S = severe; M = mild.
† SS = severe signs; NVS = no visible signs; MS = moderate signs.

moderate leukopenia. Also, both resisted challenge 3 weeks later when inoculated with the parent Bucyrus strain of virus, while controls developed typical arteritis.

Neutralization of Arteritis Virus by Convalescent Serum.—Serum from a horse inoculated with 10th kidney cell passage of Bucyrus virus and subsequently given parent virus completely inhibited the cytopathic effect (CPE), whereas serum taken from this and other horses prior to inoculation did not.

Thermal Stability.—The 44th kidney cell passage of the Bucyrus strain was tested for viability at various intervals after exposure to temperatures of 4, 37, and 56 C. The virus stored at 4 C. was viable after 75 days of storage, the longest period tested. Virus stored at 37 C. produced a CPE after 2 days' storage but not after 3 days. A CPE was produced after 20 minutes' exposure at 56 C. but not at 30 minutes.

Discussion

Arteritis virus was readily isolated in primary cultures of equine kidney cells from spleens of fetuses aborted due to natural or experimental infection. It is presumed that other organs would have yielded the virus if they had been available. Two or 3 passages were required before the virus from fetal spleen became cytopathic. This is believed to be due to the low virus content of this organ. Once isolated in kidney cell cultures, however, subsequent passages caused rapid and complete necrosis of the entire culture.

Horses with no known prior exposure were inoculated with the 7th, 10th, 25th, 46th, and 68th passage levels of the Bucyrus strain and with the 16th and 40th passage levels of the Penn strain. The 7th and 10th passages of Bucyrus virus caused severe disease. The response to passages 25, 46, and 68 was relatively mild, being milder with increased passage. All passages were able to evoke solid immunity, however, as evidenced by resistance of all horses, exposed to those passage levels of virus, to severe challenge 3 weeks after inoculation.

The 16th and 40th passages of the Penn strain of arteritis virus elicited mild disease. It should be pointed out, however, that the epizootic during which this strain originated was mild in the adult horses, especially when compared with natural infection with the Bucyrus strain.

Circulating antibody is produced following infection of horses by the arteritis virus as indicated by inhibition of CPE in cell cultures, by convalescent serum, and by lack of inhibition by preinoculation serum. These results would support the idea that a serum-neutralization test and possible other serologic procedures could be adapted for studying the arteritis virus-antibody relationship.

Arteritis virus is extremely stable at low temperatures. It remains viable for at least 6 years at −20 C., when frozen in whole organs, and for at least 75 days in tissue culture mediums at 4 C. It was viable 2 days at 37 C., and for 20 minutes but not 30 minutes at 56 C.

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Summario in Interlingua

Le Propagation de Virus de Arteritis Equin in Culturas Monostratal de Ren Equin

1. Le virus de arteritis equin esseva prendite ab tissus equin e propagate serialmente in primari culturas monostratal de ren equin.

2. Le virus produceva un effecto cytopathic que progredeva usque al complete necrose de culturas de ren equin.

3. Cavallos esseva inficite per injectiones intramuscular o intranasal de virus de racia Bucyrus post 7, 10, 25, 46, e 68 passages per cellulas renal. Le severitate del morbo experimental esseva reducite in le curso del successive passages de virus in culturas tissular. Le dece-sexte

e quarantesime passages de virus de racia Penn inficeva cavallos sin causar visibile signos de morbiditate.

4. Omne le cavallos exponite a virus propagate in histoculturas disveloppava immunitate resistente al provocation, 3 septimanas plus tarde, con le virus original de racia Bucyrus.

5. Cavallos exponite a virus disveloppava anticorpore circulante que inhibi le effecto cytopathic del virus in histoculturas.

6. Le racia Bucyrus de virus esseva viabile post 6 annos de immagasinage a −20 C in un integre splen fetal. Le quaranta-quarte passage per cellulas renal esseva viabile post 75 dies de immagasinage in un medio histocultural a 4 C, sed illo superviveva solmente 2 dies a 37 C e 20 minutas (sed non 30) a 56 C.

Studies on Hemolytic *Escherichia coli* Associated with Edema Disease of Swine. I. Separation and Properties of a Toxin of Hemolytic *Escherichia coli*

J. W. Davis, D.V.M., Ph.D.; R. C. Allen, Ph.D.; R. M. Smibert, Ph.D.

SUMMARY

An electrophoretically homogeneous fraction from a sonic extract of a hemolytic strain of *Escherichia coli* (strain 755) associated with edema disease of pigs was prepared using continuous paper electrophoresis. The fraction killed pigs weighing 60 to 80 lb. when injected intravenously. The toxic fraction produced many lesions similar to those associated with edema disease.

Agar-gel diffusion produced a similar precipitin pattern when sonic extract antiserums of 2 hemolytic strains of *Esch. coli* (755 and Powers) isolated from swine with edema disease cross reacted with soluble antigens obtained from strain 755. Sonic extract antiserum of a nonhemolytic avian strain of *Esch. coli* (2D), cross reacting against soluble antigens from strain 755, produced neither a similar pattern nor precipitins against the toxic fraction.

INTRODUCTION

The toxins of gram-negative bacteria have been widely studied.[1,2] Toxins of hemolytic *Escherichia coli* associated with edema disease of swine have been studied to a limited extent by other investigators.[3-6, 8-11, 13] Clinical manifestations and lesions found in edema disease of swine suggest that there are potent toxic substances liberated from the cells during infection.[4, 13]

Hemolytic *Esch. coli* (edema disease) toxin has not been adequately studied, since preparations of sufficient purity have been difficult to obtain. Liberation of toxin from cells has previously been accomplished by alternate freezing and thawing of the cells.[4] With this type of preparation, Gregory [5] was not able to produce typical edema disease of swine. Ajl *et al.*[1] employed continuous paper electrophoresis for the final purification of toxin from *Pasteurella pestis*.

The purpose of the present work will be to study hemolytic *Esch. coli* found associated with edema disease, and attempt to separate and isolate the toxic component.

The immunologic, chemical, and physical properties of this toxic material will be described in detail in this and succeeding reports.

Materials and Methods

Culture Methods.—Escherichia coli, strain 755, serotype 138:B81:14, isolated from a pig with edema disease, was used in this study. The strain was selected since it produced more lesions of edema disease in pigs than other strains tested. Roux flasks with 125 ml. of Bacto heart infusion agar containing 4% sterile chicken blood were inoculated with a 4-hour broth culture (heart infusion serum broth) of *Esch. coli*. The flasks were incubated for 72 hours at 37 C. The cells were harvested from each flask with 10 ml. of sterile solution of 0.85% NaCl (saline), washed 3 times with phosphate-buffered saline solution (pH 7.1) at 10 C., and centrifuged at 8,000 g for 10 minutes. The cells were resuspended in buffered saline solution. Twenty-five milliliter portions of the suspended cells were treated in a 10 kc. sonic oscillator for 30 minutes at approximately 10 C. The sonic extract was centrifuged at 13,000 g for 30 minutes at 10 C. Cell extract and all fractions were stored at −56 C.

The sonic extract was filtered through a Seitz ST-L6 filter. Sterility determinations were made on the filtered extract prior to passing the material through the curtain.

Fractionization of Sonic Extract.—The filtered extract was diluted 1:6 with barbital buffer (pH 8.6, ionic strength 0.02), and 1 ml. of 0.2% phenol red was added per 100 ml. of diluted sonic extracts.[2] The solution was passed through an elec-

Received for publication Sept. 26, 1960.

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This work was supported in part by a Research Grant E-2047 (C2) from the National Institute of Allergy and Infectious Disease of the National Institutes of Health.

trophoresis cell* at a current of 55 ma. and 620 volts. An internal cell temperature of 20 to 22 C. was maintained by passing cold buffer through the cooling plate.

Hanging strip electrophoresis** was used to investigate the electrophoretic homogeneity of the fractions recovered from continuous electrophoresis of the sonic extract. The buffer system employed was sodium barbital-barbituric acid having an ionic strength of 0.075 and a pH of 8.6. Separation was carried out at 2.5 ma. for 16 hours. The paper strips were analyzed with a scanner.†

Ten-week-old pigs of mixed breeding (Yorkshire-Duroc-Hampshire) from a disease-controlled herd were used to assay the toxicity of the crude sonic extract and all fractions collected from continuous electrophoresis. The toxin-containing extracts were administered intravenously to the pigs.

Immunologic Procedures.—Antiserums against whole cells, supernatant fluid (washing), and sonic extract were prepared by intravenous injections of extract given to rabbits. Rabbits were immunized with doubling doses of antigen given at 2-day intervals, starting with 0.25 ml. through 4.0 ml. Blood samples were obtained from all rabbits 7 and 10 days following the last injection of the series. All hyperimmune serum samples had high precipitin titers. Precipitation in agar-gel for the qualitative analysis of the fractions was carried out on plates* after the method of Mansi.[7]

Total nitrogen and nonprotein nitrogen were determined by the micro-Kjeldahl method. Toxic fraction 3 was dialyzed 24 hours against 3 changes of 0.01M phosphate-buffered saline solution at pH 7.1 in the cold. Continuous agitation of the dialyzing solution was accomplished by a magnetic stirrer.

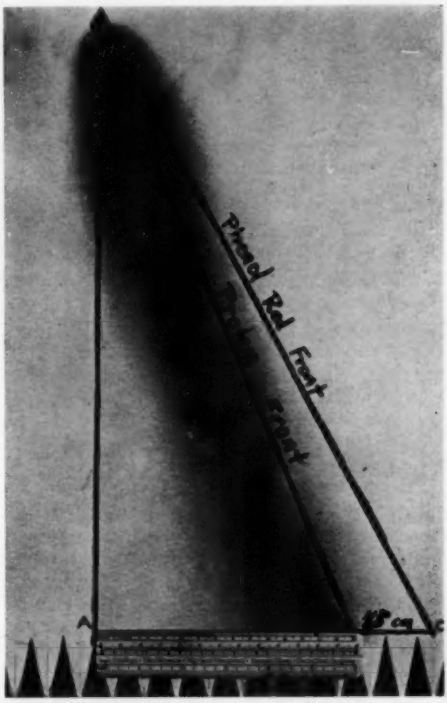

Fig. 1—Paper curtain electrophoretic pattern of refractionized toxic fraction 3 from sonic extracts of Escherichia coli strain 755, showing relationship between phenol red and protein front.

Results

Four major fractions were separated by curtain electrophoresis. Fraction 3 was the only fraction found to have toxic properties. This fraction was also the most concentrated, based on protein determinations. All the fractions were fluorescent under ultraviolet light at 260 mμ. The front of fraction 3 and the phenol red front were compared (Fig. 1). A reference point value (Rf.) for fraction 3 was calculated as the ratio of the distance between AC and AB, in which line A was a perpendicular line dropped from the point of application to a point ¼ inch above the serrated edge of the paper curtain. The Rf. value was 0.77, with a standard deviation of ± 0.05 for 3 runs of the 3 different lots of sonic extract. Fraction 3 was spread over an area on the curtain corresponding to drip points 19 through 24 (Fig. 1). The electrophoretic pattern fraction 3, using the Spinco Model R system, is shown (Fig. 2). Fraction 3, with only one major component, appeared to be electrophoretically homogeneous.

Intravenous inoculation of fraction 1 into 2 pigs, fraction 2 into 2 pigs, and fraction 4 into 3 pigs did not produce clinical manifestation of a toxic reaction. At necropsy, no gross lesions were observed. Histopathologic findings were negative. Fraction 3, however, was active when injected intravenously into 8 pigs, of which 6 died. Two to 4 mg. of protein material from fraction 3 was sufficient to kill a 60- to 80-lb. pig in 24 hours. Clinically, all the pigs developed incoordinated gait, lay on their stomachs or sides, and occasionally made bicycling movements with their feet. All pigs given fraction 3 would defecate

* Spinco Continuous Flow Electrophoresis Cell, Model CP, Beckman Instruments, Spinco Div., Palo Alto, Calif.
** Spinco Model R (Durrum Type) Electrophoresis Cell, Beckman Instruments, Palo Alto, Calif.
† Analytrol Scanner, Model R, Beckman Instruments, Spinco Div., Palo Alto, Calif.
* Plates contained 1% Ionagar No. 2 (Oxoid), produced by Oxo Limited, London, England.

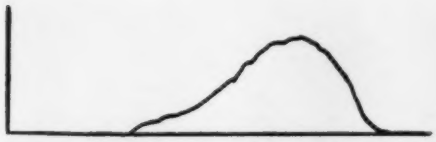

Fig. 2—Paper strip electrophoretic pattern of toxic fraction 3 from sonic extracts of Escherichia coli (strain 755), showing one major protein peak.

within half an hour after administration of the fraction. Grossly, there was slight edema in the mesentery of the coiled colon. There was severe edema of the gallbladder and at the cardiac-esophageal orifice of the stomach. The liver was friable, and the kidneys were congested. There was severe gastritis and slight enteritis. About 1 pt. of yellowish fluid was found in both the abdominal and thoracic cavities.

The following general histopathologic findings were evident. Kidneys were intensely hyperemic. The lungs had patchy hyperemia. Liver tissue contained patchy sinusoidal hyperemia with severe portal hyperemia. There was variable hyperemia and focal hemorrhage and necrosis in the lymph nodes. The gastrointestinal tract had venous vessels which were usually dilated. There was acute hyperemia in the spleen.

Two pigs inoculated with a sublethal dose of toxic fraction 3 became prostrate but eventually recovered. In some animals, inoculation of a lethal dose of toxin after recovery had little or no effect on the animals.

Inoculation of material dialyzed for 24 hours produced gastritis, edema in the mesentery of the coiled colon, and about 1 pt. of fluid in thoracic and abdominal cavities in each of 3 pigs. The lungs were edematous. One pig inoculated with a 48-hour dialysate became dull and lay on its sternum, but recovered in less than 6 hours. Gross lesions were not seen at necropsy. When the 48-hour dialysate and dialyzable material were combined and inoculated into a pig, it became prostrate for 4 hours but was eating in 20 hours. No gross lesions were seen at necropsy. The buffer solution produced no effect when injected into a pig.

Sonic extract antiserum of *Esch. coli* strain 755 has at least 2 bands against the sonic extract serum (755), but only one band against formalin-treated whole cells (Fig. 3). Fraction 3 and culture supernatant fluid (washings) produced a broad

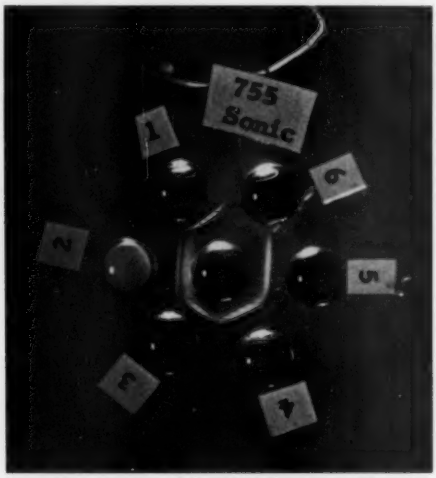

Fig. 3—Agar-gel plate showing the reaction between sonic extract antiserum from Escherichia coli (strain 755) and various antigens from strain 755. (1) Formalized washed whole cells; (2) sonic extract; (3) fraction 3; (4) refractionated fraction 3; (5) culture supernatant fluid (washings); and (6) saline control.

band which was continuous with one of the bands from the sonic extract.

Sonic extract antiserum of different strains of *Esch. coli* was compared to strain 755 antigens (Fig. 4, 5). The Powers strain, isolated from a pig with edema disease, produced a precipitin pattern in agar-gel plates similar to that of strain 755. However, there was no similarity observed in the 2D strain (2:B1) isolated from a chicken with endocarditis.* Fraction 3 of strain 755, which had toxic activity, did not react with the sonic extract antiserum of the avian strain of *Esch. coli*. However, whole sonic extract from strain 755 produced at least one band against sonic extract antiserum of the avian (2D) strain.

Discussion

The methods described provide for liberation of toxin from hemolytic strains of *Esch. coli* associated with edema disease of swine and for semipurification of the toxic fraction. Ability to reproduce the toxic fraction 3 times within an acceptable error by curtain electrophoresis indicates that it may be a suitable method for partial separation of the toxin.

* Supplied by Dr. W. B. Gross, Department of Veterinary Science, Blacksburg, Va.

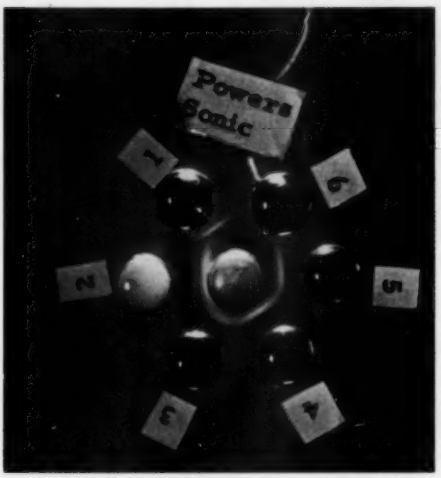

Fig. 4—Agar-gel plate showing the reaction between sonic extract antiserum from Escherichia coli (Powers strain) and various antigens from strain 755. (1) Formalized washed whole cells; (2) sonic extract; (3) fraction 3; (4) refractionated fraction 3; (5) culture supernatant fluid (washings); and (6) saline control.

Fig. 5—Agar-gel plate showing the reaction between sonic extract antiserum from Escherichia coli (strain 2D) and various antigens from strain 755. (1) formalized washed whole cells; (2) sonic extract; (3) fraction 3; (4) refractionated fraction 3; (5) culture supernatant fluid (washings); and (6) saline control.

Agar-gel diffusion was found to have little antigenic relationship to the intracellular and envelope components of a nonhemolytic coliform organism (2D) isolated from a chicken and a hemolytic coliform organism (755 and Powers) isolated from edema disease in pigs. Hemolytic coliform organisms isolated from edema disease (Powers and 755) presented a similar pattern in agar-gel in cross reactions against sonic extract antiserum.

Sweeney *et al.*[11] demonstrated that a whole freeze-thaw extract of a toxic strain of *Esch. coli* (139:B82:1) contained somatic O and X antigens. The X antigen being described as a heat-stable (100 C. for 1 hour) antigen distinct from O, K, and H antigens. The thin band observed in agar-gel plates, using sonic extract antiserum (Powers and 755) against 755 formalized whole cells and sonic extract, suggests that it may be an envelope substance loosely bound to the cell surface. Since the thin band was not seen with fraction, it probably is not part of the toxic fraction. Further investigation of the homogenicity, chemical composition, immunology, and pharmacology of the toxin is needed.

Since antiserums against sonic extracts of a strain of avian *Esch. coli* (2D) reacted only with whole sonic extracts from a strain (755) associated with edema disease and not with fraction 3, it is unlikely that all strains of *Esch. coli* contain the toxin under study, unless this toxin is nonantigenic.

Lesions similar to many of those seen in edema disease were present in pigs inoculated with fraction 3. Severity of the lesions depended to a large degree on the quantity of the material injected intravenously. A sub-lethal dose apparently gave some type of protective response, since a 2nd lethal dose of the fraction after apparent recovery of a pig had little effect upon the animal. There seems to be no explanation for this phenomenon at the present time.

A 24-hour dialysate of fraction 3 was toxic to pigs, indicating that the toxin was nondialyzable and composed of fairly large molecules. The failure of 48-hour dialysates and combined dialysate and dialyzable material to kill pigs or to produce signs similar to those of edema disease may indicate that at 24 to 48 hours at 4 C. the toxin was partially inactivated. This is also indicated by the loss of activity after storage for several days at 4 C.

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SUMMARIO IN INTERLINGUA

Studios in Hemolytic *Escherichia coli* Associate con Morbo de Edema de Porcos. I. Separation e Characterisation de un Toxina de Hemolytic *Escherichia coli*

Un electrophoreticamente homogenee fraction de un extracto sonic de un racia hemolytic de *Escherichia coli* (racia 755) associate con morbo de edema de porcos esseva preparate per medio del uso de continue electrophorese a papiro. Le fraction occideva porcos de 60 a 80 libras de peso quando illo esseva injicite per via intravenose. Illo produceva multe lesiones simile a illos associate con morbo de edema.

Diffusion in gel de agar produceva un simile configuration de precipitina quando antisero a extraction sonic ab 2 racias hemolytic de *Esch. coli* (755 e Powers) isolate ab porcos con morbo de edema entrava in reaction cruciate con solubile antigenos obtenite ab le racia 755. Antisero de extraction sonic ab un non-hemolytic racia avian de *Esch. coli* (2D), in reaction cruciate con solubile antigenos, non produceva un simile configuration ni precipitinas contra le fraction toxic.

Cultivation of Hog Cholera Virus in Subculturable Swine Buffy Coat Cells

R. W. Loan, D.V.M., M.S., and D. P. Gustafson, D.V.M., Ph.D.

SUMMARY

Studies were made of the general problem of the detection of the presence of hog cholera virus (HCV) through the use of tissue culture techniques. Two subculturable swine buffy coat cell lines were developed. The propagation of HCV in one of these cell lines was demonstrated by the reaction of susceptible pigs to the inoculation of harvested materials. A 10^{-4} dilution of supernatant fluid from a 12th tissue culture passage of virulent HCV was infective for pigs. This represented a dilution of the original stock virus of more than 10^{-21}. In 2 studies of the propagation of HCV in tissue culture, the virus was demonstrated in concentrations of 10^2 and approximately 10^5 infective doses per milliliter on the 4th day following inoculation of the cultures. Hog cholera virus was demonstrated in a persistently infected swine buffy coat cell culture after 204 days, at which time the virus was present in supernatant fluids in a concentration of approximately 10^5 infective doses per milliliter. No cytopathic effect of HCV was observed in subculturable swine buffy coat cultures.

INTRODUCTION

Although the viral nature of hog cholera has been known since 1903,[5] efforts to develop a suitable laboratory test to detect the presence of hog cholera virus (HCV) have not resulted in techniques adapted to routine use. Tissue culture techniques have been used in the study of HCV with some encouraging results. This is a report of the development of 2 subculturable lines of cells derived from swine buffy coat cells and of the study of HCV propagation in one of these cell lines.

The *in vitro* propagation of HCV was first reported by Hecke[11] in 1932. Later, Tsubaki,[22] TenBroeck,[21] Boynton,[2] Markovits and Biro,[17] Lee,[15], Frenkel *et al.*,[8] Dale and Songer,[6] Ichihara,[12,13] and Dunne *et al.*[6,7] observed that HCV could be propagated *in vitro* in a variety of swine tissues in primary cultures. A cytopathic effect of HCV was not observed by any of these investigators. Switzer[20] reported observing no cytopathic effect of HCV in subculturable lines of swine kidney, nasal mucosa, lung and swine endothelial cells, or in primary tissue cultures of swine kidney and lung. Gustafson and Pomerat,[9,10] however, observed changes in primary explant cultures which they attributed to the action of HCV. Christensen[3] also reported some cytopathic changes in tissue cultures infected with HCV. Kumagai *et al.*[14] reported that, under carefully controlled conditions, cultures of swine testicular cells exposed to HCV at the time of preparation of the cells later contained evidence of an exhalted cytopathic effect by Newcastle disease virus when exposed to this virus 3 or 4 days later.

Buffy coat cells were selected as a source of animal cells for the development of a subculturable cell line in which the propagation of HCV could be demonstrated and studied because of the ease with which they may be obtained and prepared and because they are probably free of fibroblasts. Osgood and Brooke,[18] using "gradient" culture techniques, reported the development of subculturable cell lines from human leukemic blood. More recently, Paul[19] reported the successful continuous cultivation of 4 buffy coat cell lines from normal human adult blood, and Berman and Stulberg[1]

Received for publication Aug. 15, 1960.

From the Department of Microbiology, Pathology and Public Health, School of Veterinary Science and Medicine, Purdue University, Lafayette, Ind. Submitted as Journal Paper No. 1650, Purdue University Agricultural Experiment Station.

Based on a thesis submitted by the senior author to the Graduate School in partial fulfillment of the requirements for the M.S. degree.

This investigation was supported in part by Research Grant E1516 from the National Institute of Allergy and Infectious Diseases, USPHS, and by a research grant from Eli Lilly and Company.

Presented before the Section on Research, Ninety-Seventh Annual Meeting, American Veterinary Medical Association, Denver, Colo., Aug. 14–16, 1960.

reported the continuous cultivation of 6 cell lines from normal human blood.

Materials and Methods

Tissue Culture Medium.—Eagle's basal medium, plus 10 or 20% swine serum obtained from hog cholera-susceptible pigs, 1% l-glutimine, and antibiotics, was used in developing both cell lines. Eagle's basal medium plus 10% human serum, 1% l-glutamine, and antibiotics was used in studies of HCV in cell line SBC 9/18/58. Antibiotics were used in concentrations of 100 units of potassium penicillin G per milliliter, 50 μg. of streptomycin sulfate per milliliter, 100 units of nystatin per milliliter, and 10 μg. of tetracycline HCL per milliliter.

Subculturable Swine Buffy Coat Cells.—Two subculturable lines of swine buffy coat cells were developed, although only one of these cell lines was used to demonstrate the propagation of HCV. The 1st subculturable cell line, SBC 9/18/58, was developed from buffy coat cells separated from the blood of a hog cholera-susceptible pig by the method described by Vallee *et al.*[28] with some modifications.[10] In the development of this cell line, some of the primary cultures which were subsequently pooled and subcultured were inoculated with a cell-free filtrate of medium in which Osgood's subculturable J-96 cell line, derived from a human patient with a monocytic leukemia, had been cultured for 48 hours. The 2nd cell line, SBC 2/6/59, was developed from the buffy coat cells of a normal pig without the use of a filtrate of cultures derived from human leukemic blood. Each subculturable cell line was developed from epithelioid plaques of cells which developed in the primary cultures after various periods, but which were usually apparent after 3 weeks of *in vitro* cultivation. The cells from these epithelioid plaques were subcultured after 54 days in primary culture, in the case of cell line SBC 9/18/58, and after 42 days in primary culture in the case of cell line SBC 2/6/59. The culture medium was changed 2 times each week or as required, and the cultures were subcultured at irregular intervals. Subculturable swine buffy coat cells do not tend to fall off the glass readily as the culture ages; therefore, a culture may be maintained in a flask for long periods. One culture, inoculated with HCV after 3 days, was maintained for 204 days without subculturing. Suspension of the cells in preparation for subculturing was accomplished by forcefully shaking the cells free of the glass into the fluid medium. The older cell line was subcultured 25 times in 15 months and the younger cell line 10 times in 10 months.

Virus.—Virulent, commercially available HCV and virulent HCV obtained from an infected farm herd and passed in pigs was used in this work.

Experimental Animals.—Swine used in these experiments were from the disease-evaluated swine herd at the Purdue University veterinary research farm. Pigs from the herd have been shown to be susceptible to HCV. These pigs were housed in groups of 3 in steam-cleaned and chemically disinfected, portable, screened, wooden houses placed on ground on which no pigs had been kept for at least one year.

Animal Inoculation—Each sample or dilution of the virus material to be tested was inoculated intramuscularly into each of 3 pigs housed together. Uninoculated pigs were also housed in the same area to serve as susceptible control animals. One pig in each house was given a protective dose of hog cholera antiserum. Body temperatures of inoculated pigs were taken daily for the first 11 days. Pigs obviously infected were killed at 21 days if they lived that long. The immunity of pigs given injections of antiserum and housed with infected pigs was challenged after 21 days with 1 or 2 ml. of HCV of known virulence. In one exception, the immunity of all sick pigs and healthy survivors of inoculation with material from the persistently infected culture was challenged after either 21 or 35 days. Pigs that did not sicken within 11 days following inoculation were used in subsequent experiments, or their immunity was challenged as indicated. When pigs previously given HCV were reinoculated, the pigs which had been given hog cholera antiserum were given an additional dose of serum.

Hog cholera was diagnosed on the basis of clinical signs, total white blood cell counts, and lesions at necropsy. Necropsies were performed on all pigs that died. Tryptose and brilliant green agar were inoculated with samples from the spleen. Bacteria were subcultured in standard bacteriologic mediums for identification. All material inoculated into pigs was also inoculated into thioglycollate broth. No bacterial growth was detected in this medium.

Serial Passage of Hog Cholera Virus in Swine Buffy Coat Cell Line 9/18/58.—Virulent HCV was serially passaged 12 times at 4-day intervals in cultures of cell line SBC 9/18/58. In each passage, the virus-containing fluids were diluted more than 30 times. Supernatant fluids harvested from the 2nd, 3rd, 5th, 7th, and 10th passages were tested for the presence of HCV by the inoculation of pigs with 2.0 ml. of the undiluted fluids. One milliliter of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of supernatant fluid harvested from the 12th passage was also inoculated into pigs. Supernatant fluid from an uninoculated culture was injected into pigs to demonstrate that the virus was not present in the stock cultures.

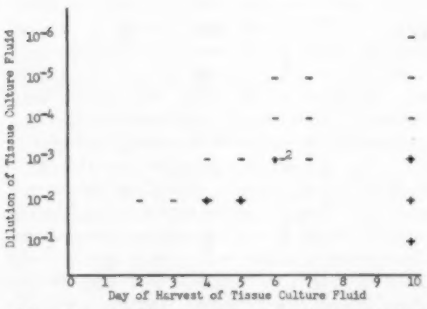
Titer of Hog Cholera Virus Following Inoculation of SBC Cultures.—Trial 1.—A 4-day-old culture in a 16-oz. bottle was inoculated with 40 ml. of a 1:10 dilution of defibrinated blood containing 10^5 pig infective doses of HCV per milliliter. This was a 5th subculture of the cell line. After incubation for 6 hours and 15 minutes, the culture was rinsed 7 times with 20 ml. of Eagle's basal medium. Fifty-five milliliters of Eagle's medium was added following the last rinse, and 5.0 ml. was harvested immediately and daily thereafter for 10 days. Twenty-five milliliters of fresh medium was added on the 5th day. Pigs were inoculated with tenfold dilutions of fluids harvested on the 2nd, 3rd, 4th, 5th, 6th, 7th, and 10th days.

Trial 2.—A 16-day-old culture of cell line SBC 9/18/58 in a 16-oz. bottle was used for this experiment. This was a 14th subculture of the cell line. It was inoculated with 30 ml. of a 1:2 dilution of fluid harvested from the 12th HCV passage in this cell line. This fluid harvested from the 12th passage had an infective titer for pigs of at least 10^4 infective doses per milliliter. After 70 minutes of incubation, the virus inoculum was removed and the culture was rinsed 7 times with 20 ml. of Eagle's basal medium. Thirty milliliters of Eagle's medium was added. One and five tenths milliliter of supernatant fluid was harvested daily for 5 days, and pigs were inoculated with 1.0 ml. of tenfold dilutions of these daily harvests.

Continuous Propagation of Hog Cholera Virus in Tissue Culture.—A 3-day-old culture of cell line SBC 9/18/58 in a 16-oz. bottle was inoculated with 60 ml. of Eagle's medium which contained less than 10 pig infective doses of virulent HCV per milliliter. Subsequently, the total volume of the culture medium was reduced to approximately 30 ml., and 15 or 20 ml. of this medium was replaced with fresh medium 2 times each week or, occasionally, once each week. Two milliliters of a 1:10 dilution of fluid harvested on the 4th day was inoculated into each of 3 pigs. One milliliter of undiluted fluid, harvested 204 days after inoculation of the culture, was inoculated into each of 3 pigs. In addition, 1.0 ml. of 10^{-3}, 10^{-4}, and 10^{-5} dilutions of this same material was inoculated into pigs.

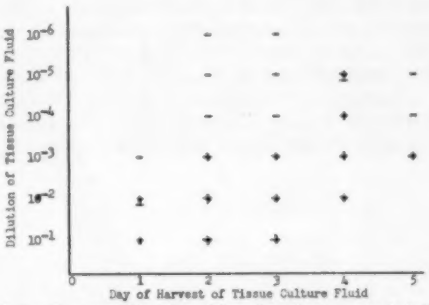
Results

Serial Passage of Hog Cholera Virus in Swine Buffy Coat Cell Line SBC 9/18/58.—Susceptible pigs inoculated with supernatant fluids harvested from the 2nd, 3rd, 5th, 7th, and 10th passages, and 10^{-2}, 10^{-3}, and 10^{-4} dilutions of fluid harvested from the 12th passage, developed typical signs of hog cholera and subsequently died. The pigs given hog cholera antiserum survived and also survived challenge. Pigs given injections of medium from cultures not previously inoculated with HCV remained healthy for 21 days. These pigs died following challenge. Gross lesions commonly associated with hog cholera were found on necropsy examination.



[1] + = 2 susceptible pigs sickened and serum-protected pig remained healthy; − = 3 pigs remained healthy. [2] Material tested in pigs twice, positive in 1st test, negative in 2nd test.

Fig. 1—Hog cholera virus titer following inoculation of tissue cultures, as determined by pig inoculation (trial 1).



[1] + = 2 susceptible pigs sickened and serum-protected pig remained healthy; ± = 1 susceptible pig sickened, 2nd susceptible pig sickened later from contact exposure, and serum-protected pig remained healthy; and − = 3 pigs remained healthy.

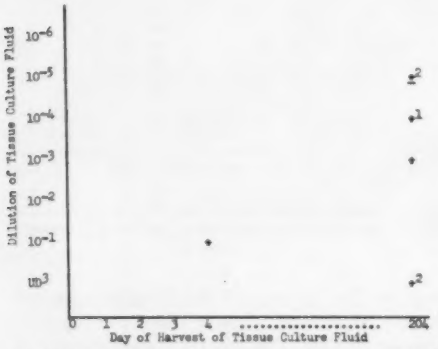
Fig. 2—Hog cholera virus titer following inoculation of tissue cultures as determined by pig inoculation (trial 2).

Titers of Hog Cholera Virus Following Inoculation of SBC Cultures—Trial 1.—The results of pig inoculation trials are shown (Fig. 1). In fluids harvested on the 4th and 5th days, there were 10^2 infective doses of HCV per milliliter. In fluids harvested on the 6th and 10th days, there were 10^3 infective doses of HCV per milliliter. A 10^{-3} dilution of fluid harvested on the 6th day caused hog cholera in the 1st inoculation trial; however, in a 2nd trial, this dilution did not infect pigs. No cytopathic effect of HCV was observed in the infected tissue culture. All pigs given hog cholera antiserum and housed with infected pigs survived the initial inoculum and subsequently survived challenge. No pathogenic bacteria were isolated from the spleens of pigs that died.

Trial 2.—The results of pig inoculation trials are shown (Fig. 2). In fluid harvested on the 1st day, approximately 10^2 infective doses of HCV per milliliter were shown. In fluids harvested on the 2nd and 3rd days, there were 10^3 infective doses of HCV per milliliter. On the 4th day, there were approximately 10^5 infective doses of HCV per milliliter in the tissue culture fluid.

On the 5th day, there were only 10^3 infective doses of HCV per milliliter. No cytopathic effect of HCV was observed in this tissue culture. All pigs given hog cholera antiserum and housed with infected pigs survived the initial inoculum and subsequently survived challenge. *Salmonella sp.* was isolated from 4 of 28 pigs that died. No other pathogenic bacteria were isolated.

Continuous Propagation of Hog Cholera Virus in Tissue Culture.—The results of pig inoculation trials are shown (Fig. 3).



[1] + = 2 susceptible pigs sickened and serum-protected pig remained healthy; ± = 1 suceptible pig sickened, 2nd susceptible pig sickened later from contact exposure, and serum-protected pig remained healthy. [2] 1 susceptible pig, as well as the serum-protected pig in each house, survived the initial inoculation and challenge with virulent hog cholera virus. [3] UD = undiluted.

Fig. 3—Continuous propagation of hog cholera virus in tissue culture as determined by pig inoculation trials.

The presence of 10^1 infective doses of HCV was demonstrated on the 4th day. On the 204th day after inoculation of the culture, approximately 10^5 infective doses of HCV per milliliter were demonstrated. One susceptible pig survived at 1.0-ml. inoculation of the undiluted fluid harvested after 204 days and also survived subsequent challenge. Only 1 of the 3 pigs inoculated with a 10^{-5} dilution of fluid harvested on the 204th day became sick within the usual incubation period of hog cholera. The 2nd susceptible pig developed signs of hog cholera 12 days later. This pig survived and withstood subsequent challenge which was delayed until 35 days following initial inoculation. Two other susceptible pigs, 1 inoculated with undiluted fluid and 1 inoculated with a 10^{-4} dilution of this fluid, survived until challenge, but they became stunted during this period. Both these pigs died following challenge. All pigs given antiserum survived the initial inoculation and subsequently survived challenge. No bacterial pathogens of swine were isolated other than one isolation of *Salmonella sp.* from a pig that died following challenge. This was 1 of the pigs that was stunted and sick at the time of challenge. No cytopathic effect of HCV was observed in this infected tissue culture.

Discussion

Although a cell-free filtrate from J-96 cells was used in early attempts to develop a subculturable swine buffy coat cell line, as in the development of cell line SBC 9/18/58, later work in the development of cell line SBC 2/6/59 showed that this was not necessary. The propagation of HCV in swine buffy coat cell line SBC 9/18/58 was demonstrated by the presence of 10^4 infective doses of virus in fluid harvested from the 12th passage. The calculated dilution of the original inoculum was more than 10^{-21}. This, together with further evidence of propagation through titration of virus-containing fluids to develop knowledge of the growth curve of the virus in tissue culture, establishes that the virus was propagated. It was also shown that the stock cultures were not infected with HCV.

The values established by titration in swine to demonstrate the growth curve of HCV in swine buffy coat cells may be influenced by the number of virus particles in the original inoculum. However, the studies establish a period of increasing titer during the first few days following inoculation of swine buffy coat cell cultures and an indication of the duration of maximum titer. Hog cholera virus not previously adapted to this tissue culture system was propagated in relatively small amounts, while HCV previously adapted to this system was propagated in considerably larger amounts. This is borne out by the higher titers found in the supernatant fluid from cultures inoculated with HCV previously passed 12 times in this cell line and by the higher titers found after 204 days in the persistently infected culture.

It appears that the earliest optimum time for harvesting HCV following the inoculation of subculturable swine buffy coat cell cultures is on the 4th or 5th day. In

the experiment on the continuous propagation of HCV in subculturable swine buffy coat cells, the virus was still present in high titer after 204 days. Two of 8 susceptible pigs survived infection with virus harvested after 204 days in tissue culture. This may have been due to increased resistance of these pigs or to reduced lethal characteristics of the virus. The pigs that survived experienced quite severe reactions as evidenced by loss of appetite, depression, and body temperatures of over 106 F.

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SUMMARIO IN INTERLINGUA

Culturation de Virus de Cholera Porcin in Subculturabile Cellulas de Coagulo Blanc Porcin

Esseva studiate le problema general de deteger le presentia de virus de cholera porcin (VCP) per medio de technicas histocultural. Duo lineas de subculturabile cellulas de coagulo blanc porcin esseva disveloppate. Le propagation de VCP in un de iste lineas de cellulas esseva demonstrate per le reaction de susceptibile porcos al inoculation de materiales recoltate. Un dilution a 10^{-4} de fluido supernatante ab le dece-secunde passage histocultural de VCP esseva infective in porcos. Isto representava un dilution a 10^{-28} del virus del stock original. In 2 studios del propagation de VCP in histoculturas, le virus esseva demonstrate in concentrationes de 10^{7} e approximativemente 10^{5} doses infective per millilitro le quarte die post le inoculation del culturas. Virus de cholera porcin esseva demonstrate in un persistentemente inficite cultura de cellulas de coagula blanc porcin post 204 dies, a qual tempore le virus esseva presente in fluidos supernatante in un concentration de approximativemente 10^{5} doses infective per millilitro. Nulle effecto cytopathic de VCP esseva observate in culturas de subculturabile coagulo blanc porcin.

Parasites of Domestic Animals in Indiana. I. The Incidence of Some Gastrointestinal Helminths of Market Weight Pigs

S. M. Gaafar, D.V.M., Ph.D.

SUMMARY

The incidence of infection with some helminths was determined by examining the gastrointestinal tracts of 318 pigs born and raised in Indiana. One or more species of parasites were found in 252 pigs (79.2%) of which 83 (26.1%) harbored stomach worms, *Hyostrongylus rubidus, Ascarops strongylina,* and *Physocephalus sexalatus;* 156 (48.4%), *Ascaris suis;* 12 (3.7%), *Macracanthorhynchus hirudinaceus;* and 165 (51.9%), *Oesophagostomum sp.*

INTRODUCTION

The list of internal parasites reported from swine is fairly extensive. Reports in the literature have concerned a wide area of North America. Dikmans [3] compiled a checklist of internal and external animal parasites of domestic animals in North America. The geographic distribution was general and no specific information was included on the incidence of helminth parasites in swine. In the southern United States, Andrews *et al.*[2] reported on the incidence of helminth parasites in swine raised in fields of small grain. A note on the parasitic fauna of Georgia by Swanson [6] included several species from pigs. The origin of the pigs was not indicated, but necropsies were performed at Moultrie, Ga. Goldsby and Todd [4] reported on the incidence of helminth infections in swine in Wisconsin and did indicate the origin. Allen [1] found that 35% of market weight swine slaughtered at Chicago were infected with *Ascaris sp.* The lack of specific information about the presence or absence of certain helminths and their incidence in Indiana prompted this investigation.

Received for publication Aug. 22, 1960.

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Submitted as journal paper No. 1655, Purdue University, Agricultural Experiment Station, Lafayette, Ind.

Materials and Methods

A total of 318 gastrointestinal tracts were collected from 175- to 240-lb. swine slaughtered at 12 small packing plants selected throughout Indiana (Fig. 1). The animals were either custom killed or purchased from farmers in the vicinity and were born and raised there. Three collections were made at each plant between Oc ober, 1958, and April, 1960.

The gastrointestinal tracts were collected in separate containers. The lining of the esophagus was stripped out and examined for imbedded parasites. Aliquot parts of 200 ml. obtained from the contents of the stomach and small intestine after dilution to 10,000 ml. were examined and all the worm parasites were counted and identified if less than 100 in number. Otherwise, only 100 worms were identified and the remainder counted. All of the large worms from the small intestine were identified and counted. The large intestines were examined for lesions and their contents were examined for helminths. Because of the large bulk of contents of the colon and cecum, no attempt was made to count or identify the species of *Oesophagostomum* or *Trichuris.*

Results

The results of this survey are limited statistically by the number of swine examined at each location and their total. However, a working base for future surveys is provided.

Three species of helminth parasites were found in the stomachs of the pigs examined. These were *Hyostrongylus rubidus, Ascarops strongylina,* and *Physocephalus sexalatus.* Two species were found in the small intestine, namely, *Ascaris suis* and *Macracanthorhynchus hirudinaceus.* Lesions caused by *Oesophagostomum sp.* were found in the wall of the large intestine and worms were found in the contents.

Two hundred and fifty-two swine (79.2%) were infected with one or more species of helminths. The incidence of infection ranged from as high as 93.3% in the northeastern area of the state to as low as 63.3% in the northwestern and east central areas. The incidence of infection in other areas was fairly uniform (Table 1, Fig. 1).

Fig. 1—Incidence of helminth infection in swine in Indiana.

Fig. 2—Incidence of stomach worms in swine in Indiana.

Infection with stomach worms was detected in all of the areas investigated; 83 animals (26.1%) were infected (Table 1, Fig. 2). The highest incidence occurred in the northeastern and the southeastern areas. The number of stomach worms varied from 50 to 6,950 per animal. *Hyostrongylus rubidus* was the most common stomach worm (72 animals or 22.6%) with 50 to 6,950 worms per animal. *Ascarops strongylina*

TABLE 1—Incidence of Helminth Infections in Indiana Pigs of Market Weight

Location	Number Examined	Number Infected	Percentage	STOMACH WORMS								Ascaris Suis		Oesophagostomum sp		M. Hirudinaceous
				Number Infected	%	Hyostrongylus Rubidus		Ascarops Strongylina		Physocephalus Sexalatus		Number Infected	%	Number Infected	%	
						No.	%	No.	%	No.	%					
Kingsbury	30	19	63.3	1	3.3	1	3.3	0	0	0	0	14	46.6	13	43.3	0
Winamac	24	19	79.1	3	12.5	3	12.5	2	8.3	0	0	14	58.3	11	45.9	0
Plymouth	28	24	85.7	5	17.8	5	17.8	2	7.1	0	0	11	39.3	19	67.9	1
Huntington	15	14	93.3	7	46.6	7	46.6	1	6.6	0	0	7	46.6	8	53.3	0
Lafayette	16	11	68.7	3	18.7	3	18.7	0	0	0	0	9	56.2	3	18.7	1
Greentown	32	27	84.4	13	43.7	13	43.7	2	6.0	1	3.1	12	37.5	20	62.5	4
Muncie	30	19	63.3	14	46.6	14	46.6	0	0	0	0	6	20.0	5	16.6	1
Clinton	28	26	92.8	7	25.0	5	17.8	4	14.2	0	0	19	67.8	21	75.0	1
Amo	25	19	76.0	10	40.0	9	36.0	4	16.0	2	8.0	14	56.0	10	40.0	1
Batesville	30	24	80.0	10	33.3	8	26.6	5	16.6	1	3.3	18	60.0	16	53.3	0
Bloomington	30	26	86.6	9	30.0	3	10.0	4	13.3	3	10.0	16	53.3	17	56.6	3
Princeton	30	24	80.0	1	3.3	1	3.3	0	0	0	0	14	46.6	22	73.3	0
TOTAL	318	252	79.2	83	26.1	72	22.6	24	17.4	7	2.2	154	48.4	165	51.9	12

occurred in 24 pigs (17.4%), with 50 to 1,900 worms per animal. *Physocephalus sexalatus* occurred in 7 animals (2.2%), each harboring 50 to 300 worms.

The large roundworm, *A. suis*, occurred in 154 pigs (48.4%) with 1 to 362 worms per animal. Nodular worms, *Oesophagostomum sp.*, as lesions in the intestinal wall or parasites in the contents, occurred in 165 pigs (51.9%), the highest incidence of any worm found.

Although no cases of severe infections with *M. hirudinaceus* were found, the infection occurred in 12 pigs (3.7%) with 1 to 17 worms per animal.

Discussion

Swine raised in Indiana seem to be infected with many of the helminth parasites that occur in other localities in the United States. The incidence of infection apparently is lower than that reported by Goldsby and Todd [4] in Wisconsin, where they found the incidence of *Hyostrongylus sp.* to be 49.5%; *Ascarops sp.*, 46.5%; *Physocephalus sp.*, 19.8%; *A. suis*, 65.4%; *Oesophagostomum sp.*, 77.2%; and *Macracanthorhynchus sp.*, 5.9%, as compared with 22.6%, 17.4%, 2.2%, 48.4%, 51.9%, and 3.7%, respectively, in the present study. Their results are in closer agreement with those of Spindler [5] and Andrews *et al.*[2] than with those of the present study.

Since parasitism usually is greater in the southern United States, the importation of swine from other states for slaughter may account in part for the apparently high incidence in Wisconsin.

It is not surprising that Allen [1] found a lower incidence of ascarid infection in hogs slaughtered in Chicago because his results were based on fecal examinations which usually give a lower incidence than direct parasite counts. Consequently, the 35% infection rate reported by Allen [1] may represent an actual incidence close to the 48.4% observed in this study.

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SUMMARIO IN INTERLINGUA

Parasitos de Animales Domestic in Indiana. I. Le Incidentia de Certe Helminthes Gastrointestinal in Porcos de Peso Mercabile

Le incidentia del infection con certe helminthes esseva determinate per examines del vias gastrointestinal de 318 porcos nate e elevate in Indiana. Un o plure species de parasitos esseva trovate in 252 porcos (79,2%). De istos, 83 (26,1% del gruppo total) habeva vermes gastric, *Hyostrongylus rubidus, Ascarops strongylina,* e *Physocephalus sexalatus;* 156 (48,4%) habeva *Ascaris suis;* 12 (3,7%) habeva *Macracanthorhynchus hirudinaceus;* e 165 (51,9%) habeva *Oesophagostomum sp.*

The *in Vivo* Response of *Mycoplasma gallisepticum* to Deoxycorticosterone Trimethylacetate

George A. Padgett, B.S., and D. E. Schoenhard, Ph.D.

SUMMARY

Four-month-old Leghorn cockerels, experimentally infected with a pleuropneumonia-like organism (PPLO) of avian origin and treated with 2 intramuscular injections of deoxycorticosterone trimethylacetate, were found to recover entirely from the infection. At the conclusion of the treatment period, no PPLO were re-recovered from the air sacs.

INTRODUCTION

Chronic respiratory disease (CRD) of poultry appears to be as much a problem as in the past, causing considerable loss to the industry. Although *Mycoplasma gallinarum*, one of the pleuropneumonia-like organisms (PPLO), can be isolated often from chickens that have CRD, not all of these organisms are pathogenic. Adler *et al.*,[1] using serologic and animal inoculation procedures, identified the virulent, pathogenic strain of *Myco. gallinarum* as S6. It has been proposed by Edward[2] that strain S6 and others possessing the characteristics of strain X95 be given the species name *gallisepticum*. The latter classification has been used in this report.

To combat the PPLO involved in CRD, research workers have tried during the last 10 years, with varying degrees of success, a broad range of chemotherapeutic agents and antibiotics.

A preliminary experiment,[3] involving the *in vitro* and *in vivo* use of deoxycorticosterone (DOC) to inhibit the PPLO of CRD, encouraged the writers to carry out more refined *in vivo* experiments. This report describes the results of one of these experiments.

Received for publication Sept. 15, 1960.

From the Department of Microbiology and Public Health, Michigan State University, East Lansing. Published as Journal Article 2680, Agricultural Experiment Station, Michigan State University.

The authors thank Ciba Pharmaceutical Products, Inc., for supplying the deoxycorticosterone trimethylacetate and diluent employed in this study.

Materials and Methods

The strain of *Myco. gallisepticum* used in this experiment was isolated in the diagnostic laboratory at Michigan State University from a bird with a field case of CRD. The culture was typical of PPLO associated with CRD, as shown by serologic, cultural, and pathogenic properties.

Prior to inoculation of the birds, the PPLO were serially passaged 8 times in the allantoic fluid of 7-day chicken embryos.

By means of a blunt 4-inch 20-gauge needle, 4-month-old Leghorn cockerels, apparently disease-free, were inoculated intratracheally with 0.5 cc. of pooled PPLO-containing allantoic fluid from 25 12-day chicken embryos. The birds were housed in isolation pens, with no possible contact between pens. They were fed a commercial, antibiotic-free, grower ration. Feed and water consumption were measured in all pens, beginning 22 days preinfection, until termination of the experiment. All birds were weighed at the beginning of the experiment and immediately before they were killed.

The experimental design is shown (Table 1). The birds in pens II, III, and IV, to be treated with deoxycorticosterone trimethylacetate (DOCT, 25 mg./cc. as commercially prepared),* were allotted to 2 subgroups per pen. Those in one subgroup were given 0.5 cc. of DOCT diluted with 0.5 cc. of a commercial diluent** at each treatment; those in the other subgroup were given 0.6 cc. of DOCT with 0.4 cc. of the same diluent. All birds were treated twice, 22 and 43 days after inoculation with the PPLO-containing allantoic fluid. The DOCT was injected into the right side of the breast, the left side serving as a control to determine the degree of absorption of the DOCT.

Twenty-one and 41 days post-treatment, respectively, half the birds in each pen were killed and examined for lesions of CRD. An attempt was made at the same time to isolate PPLO from the abdominal, diaphragmatic, and thoracic air sacs of the birds. Portions of the air sacs were excised, weighed, and placed in 1 cc. of PPLO broth (Difco) enriched with 1% serum fraction (Difco) and containing thallium acetate in a 1: 2,000 concentration. The tissue was then macerated and serially diluted to 5 places. For each dilution, 0.01 cc. was dropped on PPLO agar (Difco) enriched with 4% serum fraction and containing a 1: 2,000 concentration of thallium acetate. The

* Percorten trimethylacetate, produced by Ciba Pharmaceutical Products Inc., Summit, N.J.

** Ciba Pharmaceutical Products Inc., Summit, N.J.

plates were incubated at 37 C. for 72 hours, after which the number of organisms contained in each 0.01-cc. drop was counted at 100x magnification. From this, the number of organisms per milligram of tissue was calculated.

Results

Approximately 12,000 organisms per milligram of tissue were isolated from infected birds immediately prior to treatment (Table 2). The data (Table 2) indicate that at necropsy 21 days post-treatment, PPLO could not be isolated from 8 of the 18 birds that had been infected and treated (pens III and IV). From the other 10 birds, an average of less than 100 organisms per milligram of tissue was recovered. At the same time, an average of 5,700 organisms per milligram of tissue was isolated from the infected, nontreated control birds (pen V); all birds from this pen were found to be infected.

At necropsy, 41 days post-treatment, PPLO were not isolated from the infected, treated birds, but an average of 100 PPLO per milligram of tissue was isolated from 6 of the 9 infected, nontreated birds. At this time, PPLO were not isolated from 3 of the birds in this pen (Table 2).

Organisms could not be isolated from birds in either of the noninfected groups (pens I and II) at any time during the course of the experiment.

The number of PPLO per milligram of tissue isolated before and after treatment (Table 2) are presented in graphic form (Fig. 1).

Except for slight enlargement of the testes, no evidence of toxicosis due to DOCT was observed upon necropsy examination of any of the treated birds, infected or noninfected.

When the birds were killed prior to treatment, the typical lesions of CRD, *i.e.*, tracheitis and aerosacculitis with an accumulation of exudate in the trachea and air sacs, were marked in all the inoculated birds. Twenty-one days later, when half the remaining birds were killed, the air sacs and tracheas of all except 2 of the infected, treated birds appeared normal; the air sacs of these 2 birds were slightly cloudy, but no exudates were present. In the infected nontreated birds, lesions of CRD identical to those observed at the pretreatment killing were found.

At the termination of the experiment, when all the remaining birds were killed, all infected, treated birds appeared normal. Four of the 9 infected, nontreated birds had slightly cloudy air sacs; the rest appeared normal. Lesions of CRD were not seen in the noninfected birds during the entire experiment.

Average weight gains of the infected, nontreated birds were considerably less than in any of the other 4 groups of birds (Table 3).

All of the DOCT did not appear to be absorbed at the time the first birds were killed, 21 days post-treatment; the amount of DOCT remaining when the last birds were killed appeared considerably greater than that remaining at 21 days post-treatment.

No observable difference in effect between the 0.5-cc. and 0.6-cc. doses of DOCT was detected throughout the course of the experiment.

Discussion

It may seem strange that a mineralocorticoid should behave as an antimicrobial agent *in vivo*. In the mammal, DOC activity is limited to an influence on sodium, potas-

TABLE 1—Experimental Design

Pen No.	Bird status	Total No. birds	No. killed pretreatment	Treatment*	No. birds observed post-treatment	
					21 days	41 days
I	Noninfected, nontreated	14	3	11 birds—none	5	6
II	Noninfected, treated	23	2	11 birds—0.5 cc. 10 birds—0.6 cc.	6 5	5 5
III	Infected, treated	21	3	9 birds—0.5 cc. 9 birds—0.6 cc.	4 5	5 4
IV	Infected, treated	20	3	9 birds—0.5 cc. 8 birds—0.6 cc.	5 4	4 4
V	Infected, nontreated	18**	0	18 birds—none	9	9
Totals		96	11		43	42

* Dosage for each of 2 treatments. ** Removed from pens III and IV postinfection.

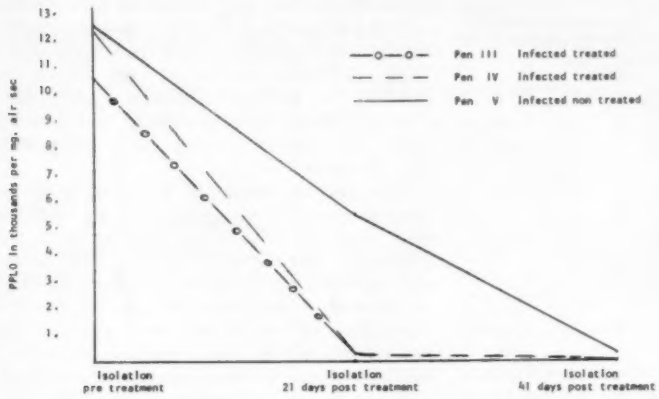

Fig. 1—Average number of organisms per milligram of air sac recovered from birds inoculated with avian pleuropneumonia-like organisms and treated with deoxycorticosterone trimethylacetate.

sium, and water metabolism,[4] any benefit accruing to an animal treated with this hormone being attributed to the "salt effect." In large doses, DOC has been shown to be toxic to mammals. However, as pointed out in the results of this study, there was no significant, observable, macroscopic toxic effect of DOCT in the birds. Boas [5] and Conner [6] have reached a similar conclusion. Like cortisone and hydrocortisone, DOC seems to behave as an anti-inflammatory agent, since the gross inflammatory lesions of the trachea and air sacs disappear with its use. It is interesting, also, that the appetite of the birds seemed to be stimulated by DOC, with a commensurate weight

TABLE 2—Number of Avian Pleuropneumonia-Like Organisms per Milligram of Air Sac Tissue Recovered from Birds with Chronic Respiratory Disease

Pen No.	Bird status	Dosage (cc.)	Pretreatment			21 days post-treatment			41 days post-treatment		
			No. birds	Total count	Count/ mg. tissue	No. birds	Total count	Count/ mg. tissue	No. birds	Total count	Count/ mg. tissue
I	Noninfected, nontreated	....	3	0	0	5	0	0	6	0	0
II	Noninfected, treated	0.5	1	0	0	6	0	0	5	0	0
		0.6	1	0	0	5	0	0	5	0	0
III	Infected, treated	0.5	1	150,000	12,300	4	0 4,500 2,000 2,200	0 200 100 60	5	0	0
		0.6	1	460,000	9,600	5	100 0 0 0 3,000	10 0 0 0 70	4	0	0
IV	Infected, treated	0.5	1	439,500	15,700	5	1,900 0 400 0 2,500	100 0 20 0 50	4	0	0
		0.6	1	290,000	9,700	4	2,000 0 900 0	60 0 50 0	4	0	0
V	Infected, nontreated	....	2	827,200 426,400	13,300 12,000	9	15,000 90,000 140,000 250,000 45,000 1,200,000 55,000 300,000 950,000	1,000 4,000 5,300 5,400 1,400 15,600 1,500 7,200 10,600	9	0 2,500 0 1,500 7,000 7,200 3,600 0 1,500	0 100 0 20 100 150 200 0 100

TABLE 3—Weight Gain of Birds Infected and Noninfected with Avian Pleuropneumonia-Like Organisms, and Treated with Deoxycorticosterone Trimethylacetate

Pen No.	Bird status	Total weight gain av. per bird (lb.)	Increase over infected, nontreated controls
I	Noninfected, nontreated	1.65	0.86
II	Noninfected, treated	1.68	0.89
III & IV	Infected, treated	1.32*	0.53*
V	Infected, nontreated	0.79	.....

* Average of pens III and IV.

gain. However, any definite conclusions about the specific action of DOC on birds must await further experimentation, since this was not the objective of the present study.

There have been several studies concerning the effect of DOC on microorganisms. Lester and Hechter [7] found that DOC inhibited the growth of a number of gram-positive bacteria, yeasts, and molds; whereas, gram-negative bacteria were generally insensitive to this hormone.

Jefferson and Sisco [8] reported that steroids inhibit the growth of *Aspergillus niger*. Maxwell *et al.*[9] found that the yeast *Saccharomyces fragilis* was almost completely inhibited by 4-androstene-3,17-dione; 1-androstene-3,17-dione; androstene-3,17-dione; DOC; and progesterone. Our previous report [3] brought to light another group of microorganisms, PPLO, that are inhibited by DOC. All of these reports pertain to *in vitro* experiments.

The fact that DOC is an effective antimicrobial agent *in vitro* suggests that it also acts directly upon the microorganisms *in vivo*. Therefore, the beneficial therapeutic activity of DOC is probably not a simple result of the physiologic response of the host. This conclusion seems to have merit in light of the work of Maxwell *et al.*,[9] who showed that 1-androstene-3,17-dione is lethal, not just inhibitory, to growing cells of *S. fragilis*.

The results recorded here, as well as those previously reported, lead us to infer that DOCT effectively inhibits *in vivo* the organisms associated with CRD. This inference is based on these assumptions: (1) the number of birds treated was adequate; (2) the strain of PPLO used was typical; (3) the vehicle of the DOCT was not itself inhibitory; and (4) the response of the birds was not due to nonspecific factors. Experiments are presently under way to test these assumptions.

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SUMMARIO IN INTERLINGUA

Le Responsa in Vivo de *Mycoplasma gallisepticum* a Trimethylacetato de Disoxycorticosterona

Gallettos del racia Leghorn de 4 menses de etate, experimentalmente inficite con un organismo pleuropneumonia-like de origine avian e tractate con 2 injectiones de trimethylacetato de disoxycorticosterona, se restabliva completemente ab le infection. Al conclusion del periodo de tractamento, nulle organismos pleuropneumonia-like esseva retrovate in le saccos de aere.

Evidence of Late Transmission of *Staphylococcus aureus* Strains Within a Divided Flock of Turkeys After 11 Weeks of Age

W. Whitney Smith, Ph.D.; Merthyr L. Miner, D.V.M.; J. Alan Thomas, D.V.M.; Paul B. Carter, Ph.D.; Yoko Yamakawa Elsner

SUMMARY

When poults were brooded together, then separated into 4 adjacent range pens, some *Staphylococcus* strains caused death in all pens; others did their major damage in one pen, but were also active in others; still other staphylococci occurred in one pen only, indicating that invasion and dissemination took place after the poults were separated from each other at age 7½ weeks.

When deaths were grouped chronologically, it appeared that certain *Staphylococcus* strains caused death throughout the entire staphylococcosis season; other strains struck early in the season and then disappeared; and still other strains were not detected early in the epornitic but occurred late, after the 17th week of life of the turkeys.

When deaths were grouped both chronologically and by pens, it was apparent that some strains were not only active for a limited time, but were limited largely to one pen.

Nothing in these data negates the earlier observation at our laboratory [2] that poults may leave the hatchery bearing the staphylococci which cause staphylococcosis on the range after the 11th week of life.

Thus, it is indicated that an epornitic of turkey staphylococcosis is not necessarily a single epornitic, but may consist of a continuing series of fatalities caused first by one strain, then by another. Where flocks are separated on the range by roads and wire only, a small epornitic may occur in one section and not affect birds in a neighboring area; other strains may cause damage throughout several adjacent range pens and throughout the entire staphylococcosis season.

Received for publication Oct. 18, 1960.

From the Department of Bacteriology and Public Health and the Department of Veterinary Science, Utah State University, Logan. Journal Paper No. 146 from the Utah Agricultural Experiment Station, Logan.

Supported by grants-in-aid of research of Abbott Laboratories, the National Turkey Federation, and the Utah Turkey Federation.

INTRODUCTION

Many strains of *Staphylococcus aureus* are involved in epornitics of primary staphylococcosis (also known as staphylococcal synovitis) in turkeys, though signs of synovial involvement are sometimes absent.[3] With the use of a bacteriophage system for typing staphylococci, specifically developed to study epizootics of this disease, it has been shown that staphylococci can be isolated from poults before they leave the hatchery in their 2nd day of life,[2] even though death does not occur until many weeks later.

Having shown that there is an early staphylococcal invasion of the turkeys, the same study [2] suggested that there may be also a later invasion. Repeated failure to cause epornitics of staphylococcosis in flocks exposed to artificially infected turkeys encouraged the idea that some factor in transmission, such as the presence of turkeys of a susceptible age, is missing in flocks of turkeys 10 weeks of age and older. The study reported here suggests that late contagion does occur.

Materials and Methods

An opportunity to explore this question was afforded by a field experiment involving 2,263 unsexed turkeys hatched February 16, 1957. These were brooded in a long narrow building as 1 flock, separated into 4 groups by wire partitions. From north to south, the groups of poults were numbered 4, 3, 2, and 1. West windows only were open; and as far as can be measured, all groups received identical treatment. The only recognizable difference in treatment in the brooder was that the major entrance was through group 1, and this section was traversed whenever the brooder was entered or left. Nothing in the results indicates

that this difference had any influence on the course of the epornitic of staphylococcosis.

At 7½ weeks (April 8), groups 1 and 2 were moved to slat pens as a prerange hardening and transition treatment. At 8½ weeks, birds of groups 3 and 4 were placed directly on the ground in range pens 3 and 4. At 9½ weeks, a heavy snow storm occurred. Group 3 and 4 birds had little protection on the range, but group 1 and 2 birds, still in the slat pens, were considerably shielded.

At 11 weeks (May 4), groups 1 and 2 were moved from the slat pens to range pens 1 and 2. On June 12, when turkeys were 15½ weeks old, range pen 1 was flooded because a gopher had broken the wall of an irrigation ditch.

The 4 field pens were separated from each other by two 8-ft. wide, wire-enclosed wagon roads running at right angles to opposing pens. Pens 3 and 4 lay northwest and northeast, respectively; pens 2 and 1, southwest and southeast. Except for the hardening-pen treatment of group 1 and 2 birds, these 4 groups were treated as 1 flock.

Unless the flood introduced *Staphylococcus* strains, none of the manipulation of the groups should have caused one pen to yield different strains of staphylococci than another. The snow storm or the slat pen treatment might have caused a different incidence of disease, but should hardly favor one *Staphylococcus* over another or introduce new strains.

The 1st death due to staphylococci was on May 3, when the birds were 11 weeks old. Every dead bird was necropsied. Within the limits of our methods cultures were secured from every turkey that died of staphylococcic infection.

In earlier studies by Smith *et al.*[2,3] the typing was done with international staphylophage 44A, grown on our "turkey" staphylococcus strain J16 and called 44AT (for turkey), and with 9 new phages designated "J" phages.

At our request in 1957, Blair* and Williams** independently studied a few of our isolates from turkeys and demonstrated that at 1,000 times the routine test concentration, "turkey" staphylococci responded to several of the international phages. All cultures in the study reported here were subjected by the technique of Blair and Carr[1] to the 9 "J" phages and 44AT at routine test dilution, and to 26 international typing staphylophages at 1,000 times routine test concentration. Of the international typing phages, several reacted with 1 or 2 cultures. Only 5, however, reacted with more than 3 staphylococci in this study and those 5 (6/42E/44A/52/54) have been added to our "J" series in defining strains; 44A was continued in our scheme as 44AT.

* John E. Blair, Hospital for Joint Diseases, New York, N.Y.

** R. E. O. Williams, Central Public Health Laboratories, London, England.

Results and Discussion

Two strains, S27 and S28 (*see* Table 1), caused substantial deaths in one pen and no deaths in the other pens. Strain S23 was absent from the 36 turkeys that died in pen 1 but caused 8 deaths in pen 3 directly northwest of it and caused a total of 17 deaths. Strain S27 killed 8 of the 60 turkeys that died in pen 3 but none in any other pen. Strain S28 killed 6 turkeys in pen 1 and none in any other pen. This is interpreted to mean that these 3 strains spread from one or possibly more carriers to other turkeys some time after the 7½-week period when they were separated from each other. Indeed, studies of the loss caused by 5 strains (S23, S24, S26, S27, and S28) suggest that these strains were communicated after 7½ weeks.

Treating the entire flock as one unit, the sequence of the appearance of the various strains (Table 2) indicated that attack, crisis, and disappearance of the Staphylococcus occurred for several of the recognized strains. Strain S21 caused the high-

TABLE 1—Distribution of Staphylococcus Strains

Phage-sensitivity pattern	Staphylococcus strain	Pen 1	Pen 2	Pen 3	Pen 4	Total
J1A/J1B/J5/J12A/J18/J21A*	S21	5	3	9	6	23
J1B/J5/J12A/J18/J21A/54**	S22	2	6	6	5	19
J1A/J1B/J5/J12A/J21A/44AT†	S23	..	5	8	4	17
J1/J1A/J1B/J5/J18/J21A	S24	8	2	2	1	13
J1B/J5/J12A/J21A	S25	1	6	3	3	13
J1/J1B/J5/J12A/42E/54	S26	4	1	1	2	8
J1B/J12A/J21A/44AT	S27	..	..	8	..	8
J1B/J21A/42E/54	S28	6	..	..	..	6
54	S29	..	1	2	1	4
J1/J1A/J1B/J5/J12A/J18/J12/J21A/52/44AT	S30	1	..	1	1	3
J1/J1A/J1B/J5/J18/J21/J21A	S31	1	..	1	1	3
52	S32	..	1	..	1	2
J1/J1A/J1B/J5/J12A/J18/J21A/6/54	S33	..	1	..	..	1
J1/J1A/J1B/J5/J12A/J18/J21A/52	S34	..	1	..	..	1
Nonsensitive (nontypable)	S99	6	12	19	14	51

* "J" indicates a Utah State University James-Jensen phage.[3] ** 6/42E/52/54 are international typing phages. † 44AT is international 44A grown on turkey staphylococcus strain 16.

TABLE 2—Distribution of Staphylococcus Strains by the Date on Which Turkeys Succumbed to Given Strains (Numbers Represent Dead Birds)

	Date, age of turkeys (weeks), and weeks of epornitic, respectively				
Strain	5/3 to 6/3 11 to 15½ 0 to 4½	6/7 to 6/18 16 to 17½ 5 to 6½	6/19 to 7/5 17½ to 19 6½ to 9	7/6 to 10/12 19 to 33 9 to 23	Total
S21	6	13	3	1	23
S22	1	..	6	12	19
S23	8	8	..	1	17
S24	1	8	3	1	13
S25	4	..	4	5	13
S26	3	1	3	1	8
S27	8	..	..	..	8
S28	..	..	3	3	6
S29	1	1	2	..	4
S30	..	..	3	..	3
S31	..	..	2	1	3
S32	1	..	..	1	2
S33	..	1	..	..	1
S34	..	..	..	1	1
S99*	17	12	8	14	51
Total	50	44	37	41	172

* Nonsensitive (nontypable).

est number of deaths between the 4th and 6th weeks of the epornitic and did little damage before or after. Strain S22 was slow to start and did its major damage chiefly after the 10th week. Great damage was done by strain S23 during the 1st 6 weeks of the epornitic and caused little harm thereafter. Similarly, strains S24, S25, S26, and S27 each had its own peculiar epornitic features. This suggests rather strongly that these various strains passed through the flock at different times, all of them after the 11th week of life of the turkeys.

TABLE 3—Distribution of Staphylococcus Strains by Pens and Date of Isolation

Staphylococcus strain	Location	5/3 to 6/3	6/7 to 6/18	6/19 to 7/5	7/6 to 10/12	Subtotal	Total
S21	Pen 1	3	2	..	..	5	
	Pen 2	1	1	1	..	3	
	Pen 3	2	6	1	..	9	
	Pen 4	..	4	1	1	6	23
S22	Pen 1	..	..	..	2	2	
	Pen 2	..	..	3	3	6	
	Pen 3	1	..	2	3	6	
	Pen 4	..	..	1	4	5	19
S23	Pen 1	..	..	..	..	..	
	Pen 2	..	4	..	1	5	
	Pen 3	6	2	..	..	8	
	Pen 4	2	2	..	..	4	17
S24	Pen 1	..	5	2	1	8	
	Pen 2	..	2	..	..	2	
	Pen 3	1	1	..	..	2	
	Pen 4	..	..	1	..	1	13
S25	Pen 1	..	..	1	..	1	
	Pen 2	2	..	2	2	6	
	Pen 3	1	..	1	1	3	
	Pen 4	1	..	..	2	3	13
S26	Pen 1	..	..	3	1	4	
	Pen 2	1	..	..	..	1	
	Pen 3	1	..	..	..	1	
	Pen 4	1	1	..	..	2	8
S27	Pen 1	..	..	..	..	..	
	Pen 2	..	..	..	..	..	
	Pen 3	8	..	..	..	8	
	Pen 4	..	..	..	..	..	8
S28	Pen 1	..	..	3	3	6	
	Pen 2	..	..	..	..	..	
	Pen 3	..	..	..	..	..	
	Pen 4	..	..	..	..	..	6

Table 2 also indicates the age in weeks at death and the duration of the epornitics. The chronology is arranged to bring approximately 50 turkeys into each of 4 periods of the epornitic. Period 1 records 50 birds dead in the 1st 4½ weeks of the epornitic (age 11 to 15½ weeks). In the next 1½ weeks, 44 turkeys died; in the succeeding 2½ weeks, 37 died. The next 41 turkeys, however, died during a period of 14 weeks (19th to 33rd week of life). This makes the damage done by strain S21 even more dramatic. In the 1st 6 weeks of the epornitic, strain S21 killed 19 turkeys; in the succeeding 16½ weeks, it killed only 4. The 8 deaths caused by strain S27 occurred in the 1st 4½ weeks of the epornitic.

In an earlier study, circumstantial evidence indicated that day-old poults left the hatchery already contaminated with the staphylococcus that later killed them.[2] The new data reported here do not negate this. Evidence is presented, however, that in addition to this early contamination, invasion and spread within the flock also occurred. Thus, the occurrence of strains S28 and S26 (Table 2) might be explained on the basis that S28 entered late, possibly on the range from some extraneous contact, and spread rapidly; strain S26 invaded the external or internal tissues of the turkeys in the hatchery, or earlier, and killed its carriers weeks later, each at a different time and in each of 4 pens (Table 3).

The date of death from the more damaging strains was recorded for each range pen (Table 3) and confirmed the late invasion concept. All 8 deaths from strain S27 occurred within pen 3 during the 1st 4½ weeks of the epornitic, before June 3. All deaths from strain S28 occurred in pen 1 and after June 13. The other strains were more cosmopolitan. It may well be that some strains were carried in such a way as to reach the entire flock, while others were carried by less mobile carriers.

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SUMMARIO IN INTERLINGUA

Evidentia de Tardive Transmission de Racias de Staphylococcus aureus Intra un Grege de Gallos de India a Etates de Plus Que Dece-Un Septimanas, post Lor Previe Subdivision

Quando pullos de India esseva covate in un gruppo e separate subsequentemente in 4 adjacente cortes, certe racias de *Staphylococcus* causava mortes in omne le cortes; altere racias faceva le plus grande damno in un del cortes sed esseva etiam active in alteres; e ancora altere racias occurreva solmente in un del cortes, lo que indica que le invasion e dissemination occurreva post que le pullos habeva essite separate al etate de 7½ septimanas.

Quando le mortes esseva gruppate chronologicamente, il esseva a notar que certe racias de *Staphylococcus* causava mortes durante le integre saison de staphylococcosis; altere racias attaccava precocemente in le saison e dispareva subsequentemente; e ancora altere racias non esseva detegite precocemente in le epornitia sed occurreva tardivemente, post le dece-septime septimana del vita del aves.

Quando le mortes esseva gruppate tanto chronologicamente como etiam secundo le cortes, il esseva a notar certe racias esseva active non solmente durante un restringite intervallo de tempore sed etiam predominantemente in restriction a un del cortes.

Nihil in iste datos denega le previe observation a nostre laboratorio que pullos pote quitar le covatorio portante le staphylococcos que causa staphylococcosis in le cortes post le dece-prime septimana del vita.

Assi il es indicate que un epornitia de staphylococcosis in gallos de India non es necessarimente un epornitia unic sed pote consister de un continue serie de occurrentias de mortes causate primo per un racia e plus tarde per un altere. In situationes in que greges es separate in le campo solmente per camminos e retes de filo metallic, un miere epornitia pote occurrer in un section e non afficer le aves in un area vicin. Altere racias pote causar damno in plure adjacente cortes e durante le integre saison de staphylococcosis.

Some Histochemical Studies of "Cloisonne Kidney" in the Male Angora Goat

Samuel W. Thompson, II, D.V.M., M.S.; Thomas R. Bogdon, B.S.; Donald H. Yost, D.V.M.

SUMMARY

The chemical composition of the pigmented homogeneous material associated with the basement membranes of the proximal convoluted tubules of the kidneys of castrated male Angora goats in "Cloisonné kidney" condition was studied histochemically. The brown pigment contained considerable inorganic mineral matter, not identifiable as iron or soluble calcium salts, and significant amounts of vicinal glycolic groups. Also found in the pigmented material were demonstrable quantities of cysteine, cystine, tyrosine, and amino groups. The material did not contain demonstrable iron, soluble calcium salts, acid mucopolysaccharides, glycogen, ethylenic linkages, preformed aldehydes, or nucleic acids. At present, the precise cause of the condition is not known.

INTRODUCTION

Light [7] reported on "Cloisonné kidney," a condition in castrated male Angora goats in which the basement membranes of the proximal convoluted tubules of the kidneys were transformed into thick wire-like structures. Grossly, the lesion spread diffusely through the cortex, ending at the corticomedullary junction. Microscopically, the tubules involved were always the proximal convoluted tubules. The distal convoluted tubules and the segments of both proximal and distal convoluted tubules which reside in the medulla were never involved in the alterations of the basement membranes. It was also reported that the wire-like structures remained unstained with hematoxylin and eosin (H and E), were periodic acid-Schiff (PAS) positive, occasionally were lightly iron-positive with the Prussian blue reaction (which indicates the presence of ferric ions [9]), could be blocked with melanin bleaches, could not be impregnated with silver stains, were not birefringent, and did not fluoresce when exposed to ultraviolet light. Light associated the existence of the condition with the frequent, but not inevitable, occurrence of hemosiderosis in the goat kidney.

Zahawi [12] reported on a similar condition which he observed in the kidneys of goats and described as existing coincidentally with renal hemosiderosis. These 2 reports constitute the only existing references to this condition in goats or in any other animal. The histochemical procedures employed to determine the chemical nature of the involved basement membranes will be described.

Materials and Methods

A search of the wet tissue files of the Pathology Division, U.S. Army Medical Research Laboratory, Fort Knox, Ky., was made for blocks of kidney tissue from Angora goats. Blocks of kidney tissue from 6 adult (5 years old or more) male goats affected with various degrees of grossly visible Cloisonné kidney were found. These had been fixed in buffered formalin (pH 7) prepared by the method recommended by Lillie.[6] Kidney tissues from an equal number of nonaffected goats of similar age and which had been fixed in a like manner also were selected to serve as control tissues. The blocks of tissues were embedded in paraffin. Serial sections from each paraffin-embedded specimen were cut at a thickness of 7 to 8 μ and individually mounted on glass microscope slides. Tissue sections were deparaffinized and studied by means of a wide variety of histochemical techniques.

Demonstration of Cell Structure.—Unstained tissue sections were mounted in glycerine and examined by conventional brightfield microscopy and polariscopy, and by fluorescent microscopy with the use of exciting light in the wave length range of 360 to 400 mμ. Tissue sections were also stained with Harris' hematoxlyin and aqueous eosin.[1]

Demonstration of Minerals.—Gomori's iron reaction[6]; Langeron's alizarin red S stain for calcium[5]; and microincineration in a muffle furnace for 30 minutes at 600 C were used. Duplicate

Received for publication Oct. 10, 1960.

From the Pathology Division, U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colo. (Thompson and Bogdon), and the Pathology Division, U.S. Army Medical Research Laboratory, Fort Knox, Ky. (Yost).

spodograms were prepared by the latter procedure. One spodogram was mounted dry and examined by polariscopy and darkfield microscopy. It was then examined by conventional microscopy while alizarin red S stain flowed beneath the coverslip by capillary action. The 2nd spodogram was stained with alizarin red S by conventional methods and mounted in synthetic resin.

Demonstration of Polysaccharides.—Alcian blue stain (short method [3]) was used, as well as the PAS leukofuchsin reaction alone and following *(a)* acetylation for 24 hours, *(b)* acetylation for 24 hours followed by deacetylation for 24 hours, *(c)* bromination for 24 hours, *(d)* phenylhydrazine for 30 minutes, and *(e)* malt diastase (1/1,000) digestion for 60 minutes at 35 C.[8] In addition, duplicate sections were stained with toluidine blue O (0.5% aqueous solution) according to the method of Kramer and Windrum, as given by Pearse.[9] One section was mounted in water after staining and immediately examined by brightfield microscopy; the 2nd section was differentiated in alcohol and mounted in synthetic resin.

Demonstration of Ethylenic Linkages.—The performic acid-Schiff reaction [6] was used.

Demonstration of Nucleic Acids.—Deoxyribonuclease * and ribonuclease* digestion and the Feulgen reaction [5] were used.

Collective Demonstration of Sulfhydryl Amino Groups, Tryptophan, Tyrosine and Histidine.—Tetrazotized benzidine coupled with *beta*-naphthol and (excepting tryptophan and histidine) dinitrofluorobenzene (DNFB) coupled with monosodium salt of 8 amino-1-naphthol-3,6-disulfonic acid (H-acid) [4, 11] were used.

Specific Demonstration of Sulfhydryl Groups (Cysteine).—The following methods were employed: mercury-orange stain,[8] modified in that N,N-dimethyl formamide was used in the preparation of the mercury-orange solution rather than toluene; DNFB coupled with H-acid on tissue sections in which the tyrosyl groups were blocked by prior treatment with diazoaniline and the amino groups were blocked by prior treatment with nitrous acid [4]; and the dihydroxy-dinaphthyl-disulfide (DDD) reaction of Barrnett and Seligman, as given by Pearse.[9]

Specific Demonstration of Disulfide Linkages (Cystine).—Sections were treated with benzoyl chloride to block tyrosyl, amino, and sulfhydryl groups. The cystine was then reduced with hydrocyanic acid, and the resultant thiol groups were demonstrated with DNFB coupled with H-acid.[5-11] In addition, a duplicate section was treated by the DDD reaction of Barrnett and Seligman for disulfide linkages, as given by Pearse.[9]

Specific Demonstration of Amino Groups.—Sections were treated with hydrogen peroxide to block sulfhydryl groups and diazoaniline to block tyrosyl groups.[5-11] The amino groups were then demonstrated with DNFB coupled with H-acid.

Specific Demonstration of Tyrosyl Groups.—Sections were treated with hydrogen peroxide to block sulfhydryl groups and with nitrous acid to block amino groups. The tyrosyl groups were then demonstrated with DNFB coupled with H-acid.[5-11]

* Supplied by General Biochemicals, Inc., Chagrin Park, Ohio.

Results

From all the goats which had lesions of Cloisonné kidney, unstained tissue sections had deposits of a greenish yellow, homogeneous material which occupied the interstitial spaces beneath the basement membranes of some of the proximal convoluted tubules in the renal cortex. This material was not birefringent and did not fluoresce. The normal basement membranes in affected kidneys and in the kidneys of control animals did not have intrinsic color in unstained tissue sections. In tissue sections from affected goats which were stained with hematoxylin and eosin, the greenish yellow, homogeneous material observed in the unstained sections was stained reddish brown and vividly outlined the involved tubules (Fig. 1). In all cases, the results of the various histochemical procedures were sufficently similar so that they may be described and discussed collectively.

The pigmented material described in the preceding paragraph did not, in our specimens, give a positive Gomori iron reaction and did not stain with alizarin red S, either before or after microincineration. In the spodograms, the pigment was observed as a thick deposit of birefringent white ash that lined the basement membranes of involved tubules (Fig. 2). In contrast, the ash at the sites of basement membranes in spodograms did stain red with alizarin red S. Diligent examination of the spodograms by darkfield microscopy did not reveal any reddish iron ash associated with the pigment deposits.

The pigment did not stain with Alcian blue. However, all basement membranes in both control and test tissue sections stained vividly with Alcian blue. The pigment gave a strong positive PAS reaction (Fig. 3) which was not adversely influenced by prior treatment of the tissue sections with phenylhydrazine for 30 minutes, bromination for 24 hours, or malt diatase digestion for 1 hour. Following acetylation, the material was PAS negative, but again was PAS positive upon deacetylation. All basement membranes in both control or test tissue sections gave reactions to the PAS procedure and its modifications which were similar to, but less intense than, those ob-

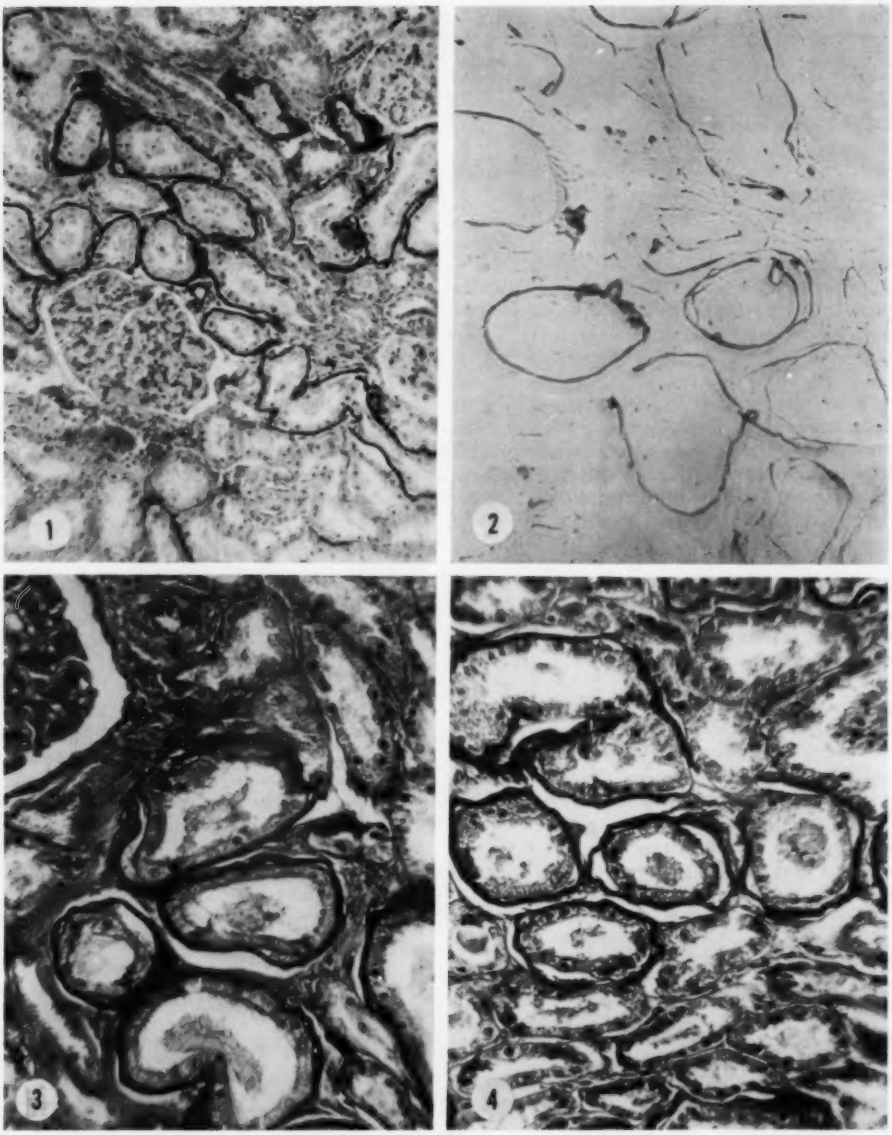

Fig. 1—Pigment beneath the basement membranes in the renal cortex of a castrated male Angora goat. H & E stain; x 90.

Fig. 2—After microincineration of a tissue section of renal cortex of a castrated male Angora goat, mineralized ash outlines the site of the pigment. Unstained, dry mount; x 180.

Fig. 3—Pigment beneath the basement membranes shown in Figure 1. Periodic acid-Schiff; x 180.

Fig. 4—Pigment beneath the basement membranes shown in Figure 1. Section stained with tetrazotized benzidine coupled with betanaphthol to demonstrate sulfhydryl and amino groups, tryptophan, tyrosine, and histidine; x 180.

served with the pigment. Neither the basement membranes or the pigment stained metachromatically with either of the techniques employed. With the preformic acid-Schiff reaction, the pigment and basement membranes gave negative results.

Ribonuclease did not digest the pigment. However, deoxyribonuclease did digest some of the components of the pigment. After digestion with the latter enzyme, the pigment deposits appeared to be somewhat deranged and lessened in quantity. The pigment gave a negative nucleal Feulgen reaction.

With tetrazotized benzidine coupled with *beta*-naphthol, the pigment beneath the basement membranes of the renal cortical tubules appeared dark reddish brown compared to the faint reddish orange of the cytoplasm and basement membranes of the tubular epithelium (Fig. 4) in both control and test tissue sections. When reacted with DNFB coupled with H-acid, the pigment appeared dark brownish purple compared to the light purple of the cytoplasm and basement membranes of the tubular epithelium in control and test tissue sections. With the procedures employed for the specific demonstration of sulfhydryl groups (cysteine), the pigment colored a faint yellowish orange with mercury-orange, and browish purple with either DNFB coupled with H-acid (Fig. 5) or DDD. The cytoplasm and basement membranes of the tubular epithelium of control and test tissue sections did not react with mercury-orange and had a weaker positive reaction to the DNFB and DDD techniques than was observed with the pigment. Similar positive results were obtained with the specific procedures employed for the demonstration of disulfide linkages, amino groups, and tyrosyl groups (Fig. 6–8). With each of the latter procedures, the pigment gave an intense positive reaction compared to less intensely positive reactions of the basement membranes and cytoplasm of the tubular epithelium, with the exception that the cytoplasm was negative for disulfide linkages.

Discussion

On the basis of the observations made with unstained tissue sections, it is evident that the pigmented material observed in Cloisonné kidney is homogeneous and possesses an intrinsic color. This intrinsic color is accentuated when the tissue section is stained with hematoxylin and eosin.

From the results obtained with the Gomori iron reaction and the alizarin red S procedure with conventional tissue sections, as well as those obtained with incinerated tissue sections, it is evident that the pigment of Cloisonné kidney does not contain demonstrable quantities of iron or soluble calcium salts. However, it does contain considerable quantities of inorganic matter, as evidenced by the extensive quantities of ash remaining after microincineration. In contrast, the ash deposited at the sites of basement membranes does contain soluble calcium salts.

Although the pigment is PAS positive, the negative results obtained with Alcian blue indicate that demonstrable quantities of acid mucopolysaccharides are not present as a component of such deposits, whereas, such polysaccharides are present in all basement membranes of both control and test tissue sections. Since the pigment is PAS positive following 24 hours of bromination, which blocks glycogen, and is not digested by malt diastase, it is felt that glycogen is not present in demonstrable quantities and therefore does not significantly contribute to the PAS positivity of the material. In addition, bromination would block any PAS positivity dependent upon carbon-carbon double bonds. The negative results obtained with the performic acid-Schiff reaction also indicates that ethylenic linkages probably do not account for the positive PAS reaction. The failure of prior treatment of the tissue section with phenylhydrazine to noticeably alter the PAS reaction of the pigment indicates that preformed aldehydes do not account for this reaction. Acetylation renders amino, hydroxyl, and vicinal glycolic groups (and in some instances, hydroxylysine and nonglucosamine saccharides) nonreactive to the Schiff reagent. The positive PAS reaction of most groups capable of being blocked by acetylation can be restored by deacetylation. This is particularly true of vicinal glycolic groups.

It is known that formalin fixation may affect certain reactive groups of protein. However, it is felt that the method of fixation employed did not significantly decrease the availability of the various reactive groups for which determinations were made.[11] The positive results obtained with the multiplicity of tests performed indicate that the pigmented material observed in Cloisonné kidney and all normal

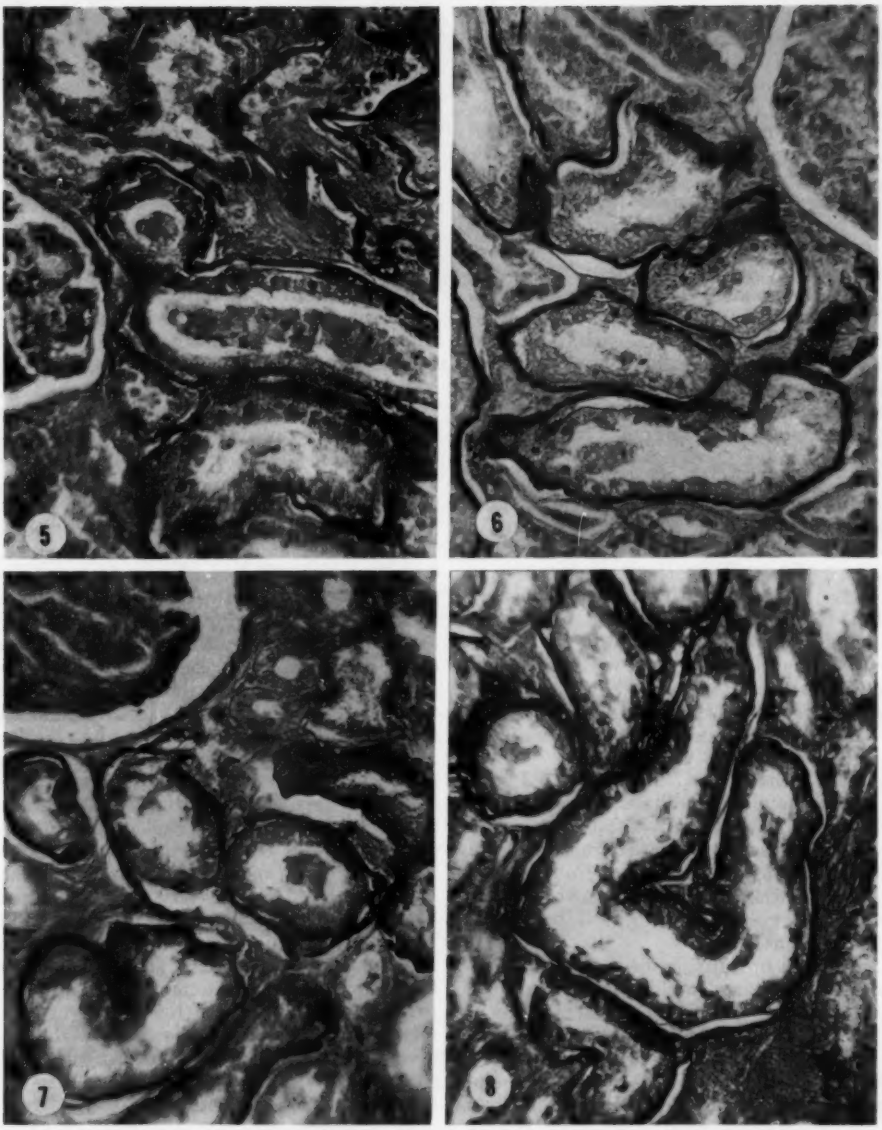

Fig. 5—Pigment beneath the basement membranes shown in Figure 1. Dinitrofluorobenzene stain (DNFB), coupled with H-acid, was employed to demonstrate the presence of sulfhydryl groups, tyrosyl groups, and amino groups; x 180.

Fig. 6—Pigment beneath the basement membranes shown in Figure 1. Section treated with benzoyl chloride to block tyrosyl, amino, and sulfhydryl groups, and treated with hydrocyanic acid to reduce cystine. The DNFB stain, coupled with H-acid, was employed to demonstrate the presence of disulfide linkages. x 180.

Fig. 7—Pigment beneath the basement membranes shown in Figure 1. Section treated with hydrogen peroxide to block sulfhydryl groups, and with diazoaniline to block tyrosyl groups. The DNFB stain, coupled with H-acid, was used to demonstrate amino groups. x 180.

Fig. 8—Pigment beneath the basement membranes from kidney of goat shown in Figure 1. Section treated with hydrogen peroxide to block sulfhydryl groups, and with nitrous acid to block amino groups. DNFB stain, coupled with H-acid, was then used to demonstrate tyrosyl groups. x 180.

basement membranes of the kidneys of the goat contain sulfhydryl, amino, and tyrosyl groups and disulfide linkages. All of these groups are characteristic of protein and, although the methods are not quantitative, the more intense reaction of the pigment as compared to the basement membranes would suggest that they are of more relative abundance in the pigment. However, the demonstration of reactive groups characteristic of protein in the pigment and basement membranes sheds no light on whether they may occur as free groups, polypeptides, or within protein molecules. Their presence only indicates that the pigment and basement membranes are composed of proteinaceous material containing cysteine, cystine, tyrosine, and amino groups.

The results obtained with ribonuclease digestion indicate that detectable quantities of ribonucleic acid are not present in the pigmented material in question. The fact that deoxyribonuclease produced some decrease in the quantity of pigment may indicate that limited quantities of deoxyribonucleic acid are present or that the enzyme was contaminated with some unknown protease. In view of the negative Feulgen reaction, the results of the tests for deoxyribonucleic acid must be considered as inconclusive.

On the basis of the results obtained with the histochemical methods employed in this study, the pigment of Cloisonné kidney, seen in castrated mature male Angora goats, can be characterized as follows:

1) It is intrinsically dark brown in fresh tissue and greenish yellow in unstained tissue sections.

2) Microscopically, it occurs as a homogeneous substance within the interstitial tissue beneath the basement membrane of adjacent proximal convoluted tubules of the cortices of affected kidneys.

3) In contrast to the basement membranes of the tubular epithelium of normal and affected kidneys, it does not stain with Alcian blue and does not contain demonstrable quantities of acid mucopolysaccharides.

4) It contains demonstrable (microincineration) quantities of inorganic matter (mineral) which are nonferrous and presumably noncalcic, or at least insoluble if calcic. In contrast, the ash at sites of basement membranes in similarly treated tissue is composed of stainable calcium salts.

5) It contains, as do the basement membranes of the tubular epithelium of normal and affected kidneys, demonstrable quantities of proteinaceous material in which cysteine, cystine, tyrosine, and amino groups are present. In contrast to the basement membranes, it gives a more intensely positive reaction to all techniques employed for the specific demonstration of such reactive groups which are characteristic of protein.

6) It contains, as do also the basement membranes mentioned previously, significant quantities of substances characterized by vicinal glycolic groups which are presumed to be polysaccharides.

7) It does not contain demonstrable quantities, if present at all, of iron, soluble calcium salts, acid mucopolysaccharides, glycogen, ethylenic linkages, preformed aldehydes, or nucleic acids.

Electron microscopy and histochemical tests for peptidases, dehydrogen esterase, and acid and alkaline phosphatases might prove of some value in the further characterization of this pigment. Facilities exist at one or the other of our laboratories to accomplish such procedures. Unfortunately, the material available for the study was not suitable for the accomplishment of such additional studies.

The cause of this peculiar condition which is intimately associated with the basement membranes of the proximal convoluted tubules in the renal cortex of the castrated male Angora goat is at present unknown. The only animals in which the condition has been seen in the United States have been procured in Texas and are said to have come from the Edwards Plateau region of that state. Little is known of the background of these animals prior to their purchase for use as experimental animals. It is known that the regions most generally used for Angora goat raising provide only a poor quality of forage for these animals.

It is not known whether the condition is reversible. From necropsy records it is known that some of the goats acquired from Texas and killed immediately have had the typical lesions of Cloisonné kidney. Others from the same shipment of animals have been held on experiment for long periods of time, sometimes up to a year, and upon necropsy have had severe lesions of the condition. In the case of the goats held on long-term experiments, the

diet usually consisted of a mixture of alfalfa hay and local grass hay, a diet quite different from that on which they were raised originally. These experiences tend to indicate that the condition is produced at the point of origin, and that once the pigment has been laid down beneath the basement membranes of the proximal convoluted tubules, the condition is irreversible.

It is not known whether any change in climate has been involved in the production of the lesions. Some Angora goats procured in Texas during the drought years were found to have the typical lesions of Cloisonné kidney. There has been no indication that the condition is on the increase or decrease at the present time.

The most likely cause of the lesion would appear to be the existence of some unusual dietary factor as yet undetermined. The coexistence in some cases of a mild renal hemosiderosis, reported by Light [7] but not observed in this more limited series of cases, perhaps may be a factor in the deposition of the homogeneous pigment. It is curious, however, that the Cloisonné condition also appears where there is no evidence of either a prior or present renal hemosiderosis. In addition, the chemical composition of the pigmented material is enough different from that of the blood pigments to raise considerable doubt as to the relationship of the 2 entities. It is also curious that a similar deposition of pigment has not been observed in the kidneys of other livestock from this region.

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SUMMARIO IN INTERLINGUA

Studios Histochimic de "Ren Cloisonné" in le Capro Angora

Le composition chimic del pigmentate material homogenee, associate con le membranas basilar del convolute tubulos proximal del renes de castrate capros angora in casos de "ren cloisonné," esseva studiate histochimicamente. Le pigmento brun contineva considerabile quantitates de inorganic materia mineral, que non esseva identificabile como ferro e non como solubile sales de calcium, e considerabile quantitates de gruppos glycolic vicinal. Esseva etiam trovate in le pigmentate material quantitates demonstrabile de cysteina, cystina, tyrosina, e gruppos de amina. Le material non contineva demonstrabile quantitates de ferro, de solubile sales de calcium, de mucopolysaccharidas acide, de glycogeno, de ligamines ethylenic, de preformate aldehydos, o de acidos nucleic. A iste tempore, le precise causa del condition non es cognoscite.

Mode of Formation of the Preputial Cavity in Domestic Animals

Madan B. Bharadwaj, G.B.V.C., B.V.Sc. & A.H., Ph.D., and M. Lois Calhoun, D.V.M., Ph.D.

SUMMARY

The penis is not free in the preputial cavity at the time of birth in most domestic animals. During embryonic development, an orderly hyperplastic invagination of the ectoderm around the urethra indicates the early fused state of the prepuce and the penis, the "balanopreputial fold."

The pattern of the fold differs in most animals. It is smoothly circular in the boar, ventrally interrupted in the dog, and sends off "secondary" folds in the region of the processus urethrae in the young male sheep and goat.

The splitting of the solid epithelial fold is accomplished by the formation of vesicles at different sites and their subsequent coalescing. The visceral epithelium lining the prepuce is formed exclusively from the inner split half of the "primary" fold. The outer split half of the primary fold and the entire secondary folds form the parietal epithelial lining. The addition of the secondary folds in the young male sheep and goat leads to the formation of an extensive, folded parietal surface of the prepuce, as compared to the smooth surface of the thin processus urethrae. This folding facilitates the passage of the glans through this region during protrusion of the penis.

In the horse, the fossa glandis forms earlier than the preputial cavity.

In the dog, the ventral mesodermal interruption of the fold leads to the formation of the "frenulum."

INTRODUCTION

Adhesions between the prepuce and the penis have been frequently reported in young male cattle, sheep, goats, and deer.[2, 7, 8, 10, 11] It has been pointed out that these adhesions are epithelial rather than fibrous.[8] These epithelial adhesions have been variously called "solid zone or ring of epithelium,"[8] "invasive epithelium,"[6] or "balanopreputial fold."[3] In the present work, the authors prefer to use the term "balanopreputial fold," because the fate of this specialized epithelium leads to the formation of the epithelial linings related to the penis ("balano") and the prepuce ("preputial"). The splitting of the balanopreputial fold is under hormonal influence and consequently it is different in castrated and noncastrated animals.[3]

Hunter[6] made some observations on the development of the prepuce in man, but his studies are not applicable to domestic animals because of their extensive preputial cavity and certain accessory structures.

Materials and Methods

For this study, specimens were collected only from killed young male animals in which the adhesions of the prepuce and penis were confirmed by gross examinations. Six species were used: horse, ox, sheep, goat, pig, and dog. Pieces of fresh tissue were removed from the regions of the processus urethrae in the lamb and kid, and from the glans and the distal part of the penis in all the animals. Tissues were fixed in Lavdowsky's mixture of formalin, alcohol, and acetic acid (FAA). Dehydration and infiltrating methods of Johnson *et al.*[7] were followed.

The following staining techniques were employed: (1) hematoxylin and eosin; (2) Weigert's and Van Gieson's for connective tissue; (3) 0.5% toluidine blue for determining the mucoid material; and (4) Mallory's trichrome stain (Crossman's modification)* to show some histochemical changes.

Hypothetical 3-dimensional model drawings were made on the basis of the outlines presented by the histologic sections at different levels.

Observations and Discussion

It is presumed that in the domestic animals, invagination of the ectoderm takes place around the urethra during the early stages of the development of the prepuce (Plate 1, Fig. 1 and 2), similar to the proc-

Received for publication Nov. 7, 1960.

From the Department of Anatomy, Michigan State University, East Lansing.

This paper was presented at the Fortieth Annual Meeting of the Conference of Research Workers in Animal Diseases, Chicago, 1959.

* Using Tissuemat, produced by Fisher Scientific Co., Pittsburgh, Pa.

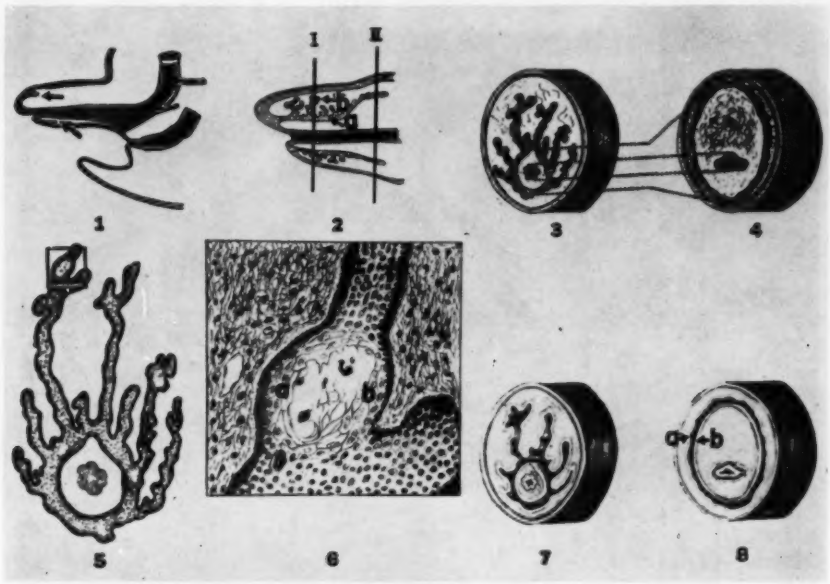

PLATE 1

Fig. 1—Developing phallus of a mammal. The arrow points at the invaginating ectoderm signifying the beginning of the formation of the prepuce.

Fig. 2—Longitudinal view of the developing processus urethrae and the glans, showing that the invaginating ectoderm follows different patterns in the regions of the processus urethrae (level I) and the glans (level II). The primary circular balanopreputial fold (a) sends off secondary radiating and branching folds toward the outer surface (b).

Fig. 3—Three-dimensional view of level I in Figure 2, showing the marked secondary folds in the processus urethrae.

Fig. 4—Three-dimensional view of level II in Figure 2, showing a relative absence of secondary folds in the glans region.

Fig. 5—The primary and secondary balanopreputial folds, showing degeneration of central cell layers and vesicle formation at various sites.

Fig. 6—An enlarged view of a vesicle (see rectangle in Figure 5). The solid epithelial mass is splitting into 2 layers of stratified squamous epithelium (a and b).

Fig. 7—The splitting is completed in the primary and secondary balanopreputial folds (level I in Figures 2 and 3). A folded preputial cavity is formed around the freed processus urethrae.

Fig. 8—The splitting is completed in the balanopreputial fold at the level of the glans (level II in Figures 2 and 4). The outer half of the fold (a) contributes to the parietal epithelial lining of the prepuce, while the inner half (b) contributes to the visceral epithelial layer of the prepuce.

ess in man.[1,6] The invaginating ectoderm, the balanopreputial fold, follows different patterns in the region of the processus urethrae and the glans (Plate , Fig. 2–4). Probably, this is due to the differences in the organizing capacity of the mesoderm in these zones. At the level of the glans, the fold surrounds the urethra in a relatively simple circular fashion (Plate 1, Fig. 2 and 4; Plate 2, Fig. 3, 5, and 6), with some species differentiation. In the region of the processus urethrae, the "primary" circular balanopreputial fold sends off "secondary" radiating and branching folds toward the outer surface (Plate 1, Fig. 2 and 3; Plate 2, Fig. 1 and 2). Near the glans, the secondary folds gradually become longer on the dorsal side of the urethra than those on the ventral side, due to the ventrally situated urethra (Plate 1, Fig. 2 and 3; Plate 2, Fig. 2). These radiating "secondary" folds do not unite with the surface ectoderm. The mesodermal mass left between the balanopreputial folds and the surface ectoderm develops into the connective tissue thickening of the prepuce, between the epidermis and the parietal epithelial lining.

Histologic and histochemical studies in

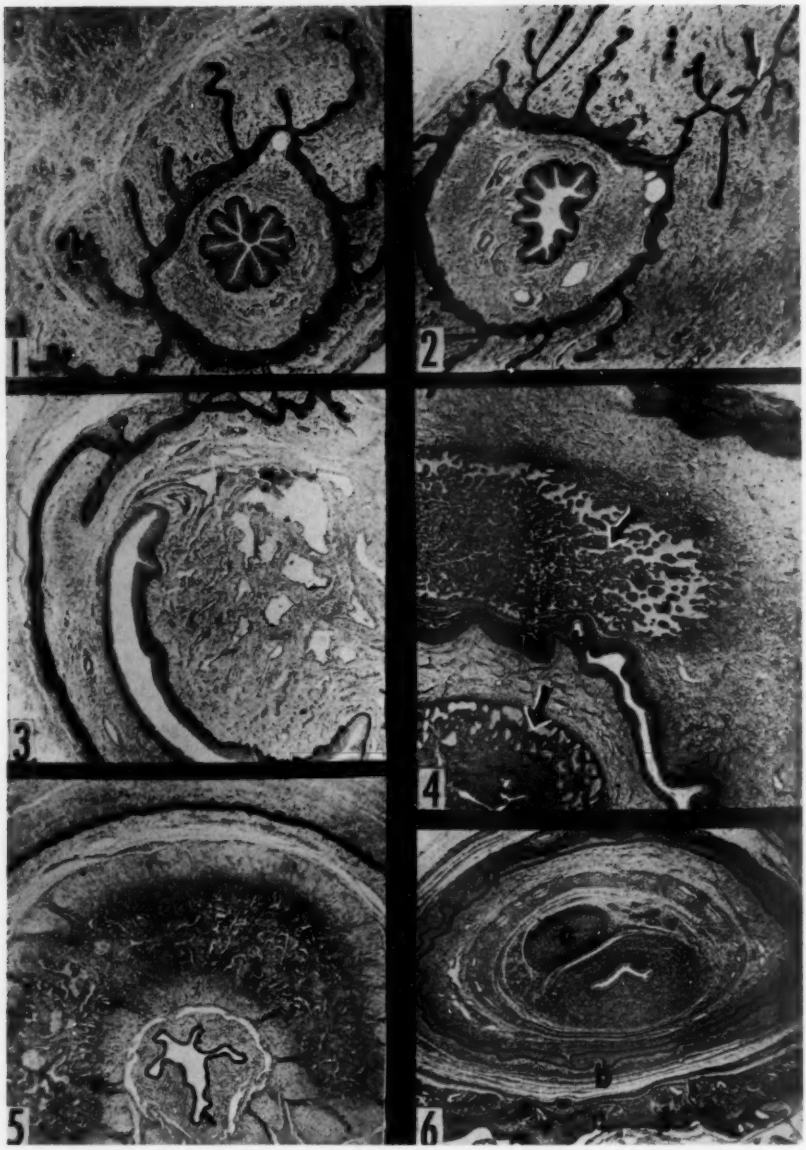

PLATE 2

Fig. 1—Processus urethrae (ram lamb) close to the distal end, showing (a) primary balanopreputial fold and (b) secondary balanopreputial folds. H & E stain; x 25.

Fig. 2—Processus urethrae (ram lamb) close to the glans, showing that the secondary folds on the dorsal aspect of the developing processus urethrae are more pronounced (arrow). H & E stain; x 25.

Fig. 3—Glans from a ram lamb, showing a relative absence of secondary folds as compared to the zone of the processus urethrae. H & E stain; x 25.

Fig. 4—Glans from a foal, showing (a) balanopreputial fold, (b) partially split epithelial fold forming a nonkeratinized, stratified, squamous lining of the fossa glandis, (c) urethral lumen. The arrows point to the cavernous bodies of the glans and the urethra. H & E stain; x 12.

Fig. 5—Glans from an immature boar, showing a relatively uniform and nonbranching balanopreputial fold. Toluidine blue stain; x 18.

Fig. 6—Glans from a pup, showing (a) balanopreputial fold, (b) "S" -shaped connective tissue interruption in the epithelial fold on the ventral aspect of the urethra, (c) developing os penis, and (d) integumentary covering of the prepuce. Trichrome stain; x 16.

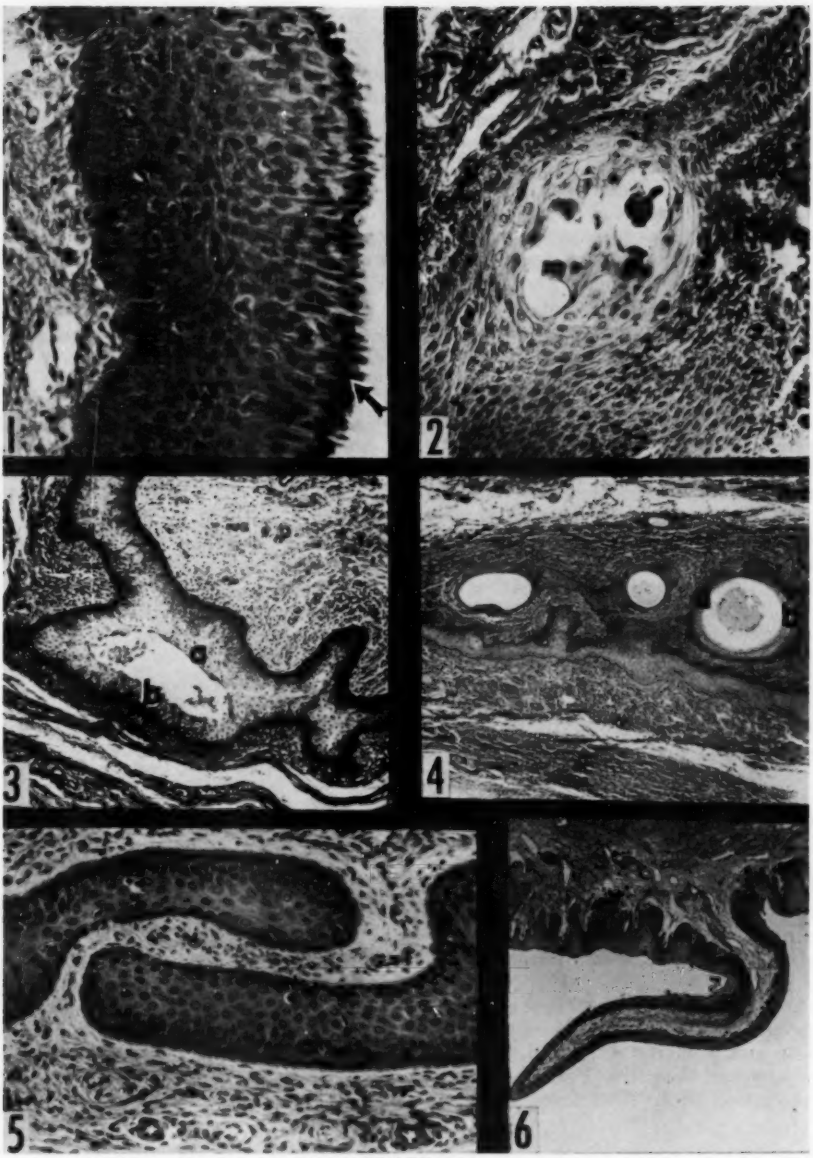

PLATE 3

Fig. 1—Glans from a young goat from which the prepuce was forcibly removed prior to the formation of the preputial cavity. The columnar cells on the free surface (arrow) present a misleading picture of the epithelial covering of the glans. After the splitting of the balanopreputial fold, these cells would have formed the basal layer of the stratified squamous epithelium of the parietal layer of the prepuce in later life (see Figure 3). H & E stain; x 350.

Fig. 2—Balanopreputial fold from a ram lamb, showing degeneration of the cells of the central layers and formation of a vesicle. Trichrome stain; x 250.

Fig. 3—Balanopreputial fold from a ram lamb, showing the progressive degeneration of the central cells and coalescence of the adjoining vesicles resulting in 2 separate layers of stratified squamous epithelium (a & b). Trichrome stain; x 110.

Fig. 4—Balanopreputial fold from a calf, showing the vesicles and the resultant formation of the 2 layers of stratified squamous epithelium of the prepuce (a & b). Trichrome stain; x 80.

Fig. 5—Glans from a pup, showing an "S"-shaped mesodermal primordium of the frenulum interrupting the balanopreputial fold ventral to the urethra. Trichrome stain; x 350.

Fig. 6—Glans from an adult dog, showing the frenulum consisting of a central connective tissue core covered by a stratified squamous epithelium. H & E stain; x 20.

different species reveal important cytologic changes in the central cell layers of the primary and secondary folds. At localized areas in the folds, the central cells swell and undergo cytolysis; and a few keratinized cells, which subsequently liquefy, may be seen. With increasing cytolysis, fluid starts to accumulate at these sites and small vesicles are formed with a few desquamated cells in them (Plate 1, Fig. 5 and 6; Plate 3, Fig. 2). The tension created by the increasing fluid of the vesicles splits the solid epithelial mass into the 2 layers of stratified squamous epithelium (Plate 2, Fig. 4; Plate 3, Fig. 3 and 4). This splitting persists in the primary and secondary folds as the vesicles continue to coalesce. The primary balanopreputial fold thus splits and its inner half contributes the entire visceral epithelial lining of the preputial cavity. The outer split half of the primary and all of the secondary folds provide the parietal epithelial lining of the preputial cavity (Plate 1, Fig. 7 and 8). This explains how the presence of the secondary folds in the region of the processus urethrae results in an extensive preputial cavity. The importance of this extensiveness is apparent when the large parietal surface of the preputial cavity unfolds in this region to give passage to the glans during erection and protrusion of the penis.

The mode of separation of the penis and prepuce is the same in all animals, but the extent of the separation appears to vary in different species. In most ruminants, the vestigial solid epithelial balanopreputial fold can still be observed at the base of the preputial cavity in the adult uncastrated male.

The keratinization of the surface epithelial cells is not observed until the splitting is complete and a cavity is formed, allowing the air to reach the cells (Plate 2, Fig. 4).

Examination of sections from the horse showed that the unfolding and keratinization of the epithelial lining of the fossa glandis is completed before the separation of the prepuce and penis has even started (Plate 2, Fig. 4).

In the pig, due to the absence of any processus urethrae or a prominent glans penis, the balanopreputial fold is smoothly circular and no secondary folds are formed (Plate 2, Fig. 5).

In the dog, the continuity of the balanopreputial fold is interrupted ventral to the urethra. An "S"-shaped mass of mesoderm is left between the separated ends of the solid primary fold (Plate 2, Fig. 6; Plate 3, Fig. 5). As the fold splits and the penis is freed, the "S"-shaped mesodermal mass still retains a thin ventral attachment between the penis and the prepuce. Eventually, it is covered by stratified squamous epithelium on either side as the visceral and parietal epithelial linings become confluent. The resulting epithelium-covered band is the "frenulum" (Plate 2, Fig. 6).

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SUMMARIO IN INTERLINGUA

Le Modo de Formation del Cavitate Preputial in Animales Domestic

Le pene non es libere in le cavitate preputial al tempore del nascentia in le majoritate del animales domestic. Durante le disveloppamento embryonic, un ordinate invagination hyperplastic del ectoderma circum le urethra indica le precoce stato fusionate de preputio e pene, le "plica balanopreputial."

Le configuration del plica differe a generalmente parlar inter animales de differente species. Illo es lisiemente circular in le porco, ventralmente interrumpite in le can, e providite de extensiones de plicas "secundari" ad in le region del processo urethral in juvene oves e capros.

Le fission del solide plica epithelial es effectuate per le formation de vesiculas in varie sitos e per le subsequente coalescentia de illos. Le epithelio visceral que revesti le preputio es formate exclusivemente ex le findite medietate interior del plica "primari." Le medietate exterior del plica primari e le integre plica secundari forma le revestimento epithelial parietal. Le addition del plicas secundari in le juvene ove mascule e in le capro resulta in le formation de un extense e plicate superficie parietal del prepution, per contrasto con le lisie superficie del tenue processo urethral. Iste plication rende plus facile le passage del glande a transverso iste region durante le protrusion del pene.

In le cavallo, le fossa del glande se forma plus precocemente que le cavitate preputial.

In le can, le interruption mesodermal ventral del plicas resulta in le formation del "frenulo."

The Partial Reactivation of Formalin-Inactivated *Clostridium botulinum* Type C Toxin

Edward W. Marty, Jr., Ph.D.

SUMMARY

The problem of toxin reappearance in *Clostridium botulinum* type C toxoids containing added sodium bisulfite to neutralize the formaldehyde was studied. Potential toxicity was demonstrated when both inactivated dialysate cultures and toxoids were neutralized and reincubated at 37 C. That formalin-resistant clostridial spores and their subsequent toxin production in neutralized products were not the cause of toxin reappearance was shown by the complete lack of viable organisms following normal formalization. Similarly, the addition of thimerosal produced no effect on potential toxicity, again indicating a source of toxin other than clostridial growth. It was also shown that neutralization alone was incapable of stimulating toxin reactivation. Increased temperatures were apparently required, since neutralized samples held at 5 C. remained nontoxic until incubated at 37 C.

Cell-free toxin studies revealed a true toxin-reactivation phenomenon. Quantitative titrations in mice showed that reactivated liquors may possess as much as 10 times more lethal toxin than formalin-containing samples taken at the same stage of inactivation. It was also shown that the reaction eliminating potential toxin reactivation followed closely behind that of normal inactivation, and that a constant relationship may exist between the 2 reactions.

INTRODUCTION

Formaldehyde inactivation of botulinum toxin has long been recorded in the literature [4,9] and, heretofore, the aspect of potential toxin reactivation after formalization has been given little consideration. Workers in related biological sciences, however, have studied reactivation phenomena and, within the virus field, there are several instances of reversible loss of infectivity following chemical treatment of viral agents. Heicken and Spicher [3] have demonstrated bacteriophage reactivation by interrupting the formaldehyde reaction with excess bisulfite, serum, or amino acids. In this instance, neutralized-inactivated phage required incubation at 37 C. before reactivation was demonstrable. Similar results have been reported by Sinkovics [8] in experiments involving the effect of $HgCl_2$ on influenza virus and by Ross and Stanley [7] in experiments with tobacco mosaic virus. It is the purpose here to report that, under specified conditions, it was also possible to partially reactivate formalin-inactivated botulinum type C toxin.

The impetus for this study arose during the development of a *Clostridium botulinum* toxoid–live virus combination vaccine for use in the veterinary field. Such a vaccine would require that any excess (unbound) formaldehyde in the toxoid be neutralized in order not to kill the live virus upon combination of the fractions. The feasibility and practical aspects of such a combination have been demonstrated.[1] After continued experience with neutralized *botulinum* toxoids, however, it was found that certain toxoid lots were toxic in the field even after extensive safety testing. The investigations which followed led to the observations on toxic reactivation which are reported here.

Materials and Methods

Production and Inactivation of Toxin.—Clostridium botulinum type C, strain "South Africa," was grown for 7 days at 37 C. in the medium of Cardella *et al.*,[2] using a dialysis system similar to that described by Sterne and Wentzel.[9] The normal toxin content of dialysate cultures grown under these conditions was $10^{5.5}$ LD_{50}/ml. or greater as tested intraperitoneally in mice. In later studies, cell-free toxin liquors were obtained by centri-

Received for publication Sept. 7, 1960.

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The technical assistance of Mrs. Donna J. Schultz is acknowledged.

fuging the dialysate culture and then filtering the supernatant fluid through an 02 Selas filter.

Toxin inactivation was achieved by adding 0.5% commercial formalin (v/v) and incubating at 37 C. When desired, toxoids comparable to commercial products were prepared by diluting the dialysate culture 1:4 in physiologic saline solution and formalizing at 37 C. for 10 days. A sterile 20% potassium aluminum sulfate solution, equal to the original culture volume, was then added as an adjuvant.

Neutralization of Formaldehyde.—The excess formaldehyde contained in inactivated toxin preparations was neutralized by the addition of a stoichiometric 50% excess of filter-sterilized sodium bisulfite. It has been shown by Taylor and Maloney [10] that formalized polio vaccine neutralized in this manner contains less than 0.6 p.p.m. of formaldehyde; hence, it was assumed that formalin activity was inhibited if a 45 to 50% excess of $NaHSO_3$ could be detected. This was tested by using a standard iodine solution and starch indicator. The standard procedure for neutralization was to add 1.1 ml. of a 10% $NaHSO_3$ solution for every 10 ml. of sample. A similar quantity of sterile saline solution was always added to non-neutralized samples when conducting comparative toxicity titrations.

Toxicity Determinations.—Qualitative toxicity was determined by intraperitoneal injection of 0.5-ml. samples into 15- to 18-Gm. mice. In many instances, toxicity tests were duplicated by subcutaneous inoculation of 1.0-ml. samples into 300- to 500-Gm. guinea pigs.

Quantitative toxin titrations, however, were conducted in mice only. Samples to be titrated were diluted in sterile phosphate buffer (pH 6.5) containing 0.2% gelatin,[8] and the diluted samples were inoculated intraperitoneally into groups of 5 mice each. Decimal dilutions and injections of 0.1 ml. were used for samples containing large amounts of toxin; whereas, doubling dilutions and 1.0-ml. inoculations were used for samples of low toxin content. All animals were observed for 7 days, and LD_{50} end points were calculated by the method of Reed and Muench.[6]

Bacteriology.—Isolation and detection of *Cl. botulinum* was achieved by inoculating 0.1-ml. samples directly into the bottom of deep, semisolid agar tubes containing a modification of a medium used by Treadwell *et al.*[11] for obtaining rapid germination of *Cl. botulinum* type A spores. The isolation medium contained yeast extract (1.0%), dextrose (0.1%), K_2HPO_4 (0.2%), and agar (0.1%) incorporated into heart infusion broth prepared from Difco dehydrated beef heart. After autoclaving or heating to expel oxygen, 0.2 ml. of a filter-sterilized 5% $NaHCO_3$ solution was added to each 10.0 ml. of medium before introduction of the sample. The inoculated medium was then incubated at 37 C. for at least 10 days before discarding as negative.

Anaerobic spore counts were determined by the tube dilution technique, using the previously described medium. Spore count samples were first heat-shocked at 85 C. for 15 minutes and then diluted decimally in saline solution. One-tenth milliliter of each dilution was then introduced separately into each of 2 tubes of medium, and the inoculated medium was incubated at 37 C. Readings were taken at 3, 5, and 7 days, and the bacterial count per milliliter was taken as being 10 times the highest dilution at which growth occurred.

Results and Discussion

It was first necessary to establish that type C *botulinum* toxin was the cause of mortalities attributed to toxoids which had been previously tested and shown to be nontoxic. Three incriminated toxoid samples, each of which was toxic for both mice and guinea pigs, were combined at a ratio of 4 to 1 with *Cl. botulinum* type C specific antitoxin.* After incubation for 3 hours at room temperature, the samples were tested for toxicity in mice. No toxicosis was observed, and it was concluded that type C toxin was present in the samples. Numerous tests have also repeatedly shown that the presence of a 50% excess of $NaHSO_3$ in toxoid is in itself nontoxic.

Several hypotheses were forwarded to explain the reappearance of toxin in known nontoxic toxoids: (1) the possibility of spore survival during formalization and their growth and production of toxin in neutralized toxoid; (2) the possibility also existed that unlysed cells were "protecting" the toxin from formaldehyde action and that active toxin was released following neutralization; and (3) the possibility of true toxin reactivation.

Whole-Culture Toxin Studies.—A dialysate culture was prepared according to the method described previously. The material was diluted 1:4 in saline solution and then formalized for 10 days at 37 C. Half of the inactivated material was retained per se, and the remainder was further processed into a complete toxoid by adding adjuvant. Bacteriologic and toxicologic tests were performed on the products, both pre- and postformalization and after formaldehyde neutralization. Neutralized products were tested after holding under several different conditions in order to determine the possible effect of temperature and time of neutralization on the resulting toxicity.

* *Clostridium botulinum* type C specific antiserum was obtained from Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

A summary of the results is presented (Table 1).

It was first observed that viable spores were not present in either the inactivated dialysate culture or the toxoid, even though the original material contained at least 10^4 spores per milliliter; *e.g.*, compare A_1 and A_2, Table 1. Similarly, the formalin treatment was sufficient to detoxify the toxin as tested by a standard mouse or guinea pig test (A_2 and B_1, Table 1). However, if the detoxified samples were neutralized with $NaHSO_3$ and reincubated at 37 C., they were again toxic (A_4 and B_3, Table 1).

Since *Cl. botulinum* was not present in either the formalized or the neutralized products, the reappearance of active toxin could not result from the regrowth of clostridial organisms. This conclusion is even more justified when one considers that added thimerosal* had no effect on the potential toxicity of neutralized samples and that spores, if present, could easily be recovered by the isolation technique (B_4 and B_5, Table 1).

The effect of temperature on toxin reappearance is also of interest. Samples neutralized and tested immediately or held at 5 C. were not toxic, yet if the same samples were then held at 37 C. for 5 days, they were again toxic (A_5 and A_6, B_2 and B_3, Table 1). Evidently, temperature acts as a catalyst in the reaction[s] causing the reappearance of active toxin. A similar requirement for bacteriophage reactivation has been recorded previously.[3]

Cell-Free Toxin Studies.—The foregoing results served to eliminate only one of the possible explanations existing for the reappearance of toxin. The aspects of toxin extraction from unlysed cells and true toxin reactivation were still eminent. If the latter phenomenon occurs, however, it should be possible to demonstrate reactivation in cell-free toxin liquors.

A cell-free preparation having a toxin content of $10^{5.38}$ mouse LD_{50}/ml. was prepared from a dialysate culture containing $10^{5.5}$ LD_{50}/ml. Formalin (0.5%) was added to the undiluted cell-free liquor, and it was placed at 37 C. At various time intervals, samples were aseptically removed for quantitative toxicity tests. At each interval, 1 sample was titrated immediately for toxicity and a 2nd sample was neutralized, reincubated for 48 hours at 37 C., and then titrated. Bacteriologic tests for clostridial growth and contamination were conducted throughout the entire experiment, and in no instance were viable organisms detected visually or bacteriologically.

The results (Fig. 1) clearly show that partial toxin reactivation occurred upon neutralization and reincubation of the formalized material. Under the conditions tested, it was possible to continually demonstrate about 10 times more toxin in neutralized samples than in formalin-containing preparations and after 12 days of inactivation, formalized liquors contained no active toxin, whereas, neutralized-reincu-

TABLE 1—Bacteriologic and Toxicologic Studies on $NaHSO_3$ Neutralized-Formalin Inactivated Dialysate Culture Toxin and Toxoid Prepared from Clostridium botulinum Type C

Sample treatment	Toxicosis (deaths/inoculated)	Recovery of *Cl. botulinum*
A. INACTIVATED DIALYSATE CULTURE TOXIN		
1. Preformalization.	$5/5^{M*}$	Positive; approximately 10^4 spores/ml.
2. Postformalization.	$1/5^{M}$ (nonspecific death; retest showed no toxicity).	Negative
3. Neutralized—no reincubation.	$0/5^{M}$	Negative
4. Neutralized—reincubate 5 days at 37 C.	$5/5^{M}$	Negative
5. Neutralized—reincubate 7 days at 5 C.	$0/5^{M}$	No test
6. Neutralized—reincubate 7 days at 5 C., then 37 C. for 5 days.	$5/5^{M}$	No test
B. TOXOID		
1. Toxoid—no treatment.	$0/5^{M}$, $0/3^{GP}$	Negative
2. Neutralized—reincubat 5 to 7 days at 5 C.	$0/5^{M}$, $0/3^{GP}$	Negative
3. Neutralized—reincubate 4 to 5 days at 37 C.	$4/5^{M}$, $3/3^{GP}$	Negative
4. Neutralized—1:10,000 thimerosal added; reincubate 4 to 5 days at 37 C.	$4/5^{M}$	No test
5. Neutralized—0.1 ml. spore suspension added,† reincubate 4 to 5 days at 37 C.	$2/5^{M}$, $3/3^{GP}$	Positive

* Superscript M = mouse toxicosis; GP = guinea pig toxicosis.

† Washed, nontoxic, spore suspension of *Cl. botulinum* type C (strain "South Africa"), containing 10^6 spores/ml.

* Merthiolate, Eli Lilly & Co., Indianapolis, Ind.

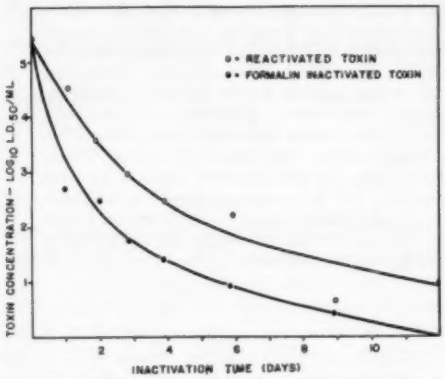

Fig. 1—The partial reactivation of formalin-inactivated Cl. botulinum type C toxin. Experiments were conducted on cell-free culture liquors.

bated material still contained about 10 mouse LD_{50}/ml. To further demonstrate that the increase in toxicity of reactivated products was true toxin activity, a neutralized-reincubated sample taken at the 48th hour during the foregoing experiment was combined at a ratio of 10 to 1 with type C specific antitoxin. No toxicity was recorded following specific serologic neutralization; hence, all lethal characteristics were attributed to the presence of true toxin.

The necessary additional inactivation time required to completely eliminate the reactivation phenomenon under the conditions of the foregoing experiment is not known. In continued study of toxin reactivation, however, it was found that this characteristic was lost after 4 to 8 weeks' holding at 5 C. in the presence of formalin. The reaction eliminating potential toxin reactivation apparently follows closely behind that of normal inactivation and, as shown (Fig. 1), a constant relationship is suggested for the 2 reactions. If the logarithm of toxin concentration is plotted against the logarithm of inactivation time, one obtains 2 nearly parallel straight lines. This suggests even more that a constant may exist. No further attempts have been made to prove the existence of such a constant at this time. Whatever the true relationship between these reactions, it can be concluded that partial reactivation of formalin-inactivated type C *botulinum* toxin is possible. These studies also emphasize the need to recognize the potential danger of formalin-inactivated products, either toxoids or killed-virus vaccines in which the unbound formaldehyde is neutralized following inactivation.

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SUMMARIO IN INTERLINGUA

Le Reactivation Partial, post Inactivation per Formalina, de Toxina de *Clostridium botulinum* Typo C

Esseva studiate le problema de re-apparition de toxina in toxoides a *Clostridium botulinum* typo C que contine un addition de sulfito de natrium pro neutralisar le formaldehydo. Toxicitate potential esseva demonstrate quando tanto le inactivate culturas dialysate como etiam le toxoides esseva neutralisate e re-incubate a 37 C. Que sporas clostridial de genere resistente

contra formalina e lor subsequente production de toxina in neutralisate productos non esseva le causa del re-apparition de toxina esseva demonstrate per le complete absentia de viabile organismos post le formalinisation normal. Similemente, le addition de thimerosal produceva nulle effecto super le toxicitate potential, lo que indica de novo un fonte de toxina altere que un crescentia clostridial. Esseva etiam provate que neutralisation per se sol esseva incapace de stimular le reactivation de toxina. Elevate temperaturas esseva apparentemente requirite, proque neutralisate specimens tenite a 5 C remaneva nontoxic usque illos esseva incubate a 37 C.

Studios de toxin acellular revelava un genuin phenomeno de reactivation de toxina. Titrationes quantitative in muses monstrava que reactivate liquores pote posseder usque a 10 vices plus toxina letal que specimens a contento de formalina, ben que le duo es prendite al mesme stadio de inactivation. Esseva etiam monstrate que le reaction eliminante le reactivation potential de toxina sequeva vicinissimemente le reaction del inactivation normal e que le existentia de un relation constante inter le 2 reactiones es integremente possibile.

Production of Foot-and-Mouth Disease Virus with High Complement-Fixing Antigenicity

Richard E. Patty, Ph.D., and Neil L. Norcross, Ph.D.

SUMMARY

A method was devised for rapid production of foot-and-mouth disease virus with high complement-fixing antigenicity in bovine kidney, swine kidney, and lamb testis cells grown on glass. Antigen titers of 1:275 were obtained from the cell portion of cultures 6 hours after addition of virus to the cells. A titer of 1:160 was obtained from the fluid portion 8 hours after addition of virus to the cells.

INTRODUCTION

The use of the complement-fixation (CF) test for the detection of foot-and-mouth disease virus (FMDV) in field samples attained more acceptance as the result of the work of Traub and Möhlmann in 1943.[14] Since then, numerous confirmatory publications have appeared.[2, 3, 9, 11, 13] Vesicular fluid and tissues of animals infected with foot-and-mouth disease (FMD) have been used as antigens to detect antiviral immune bodies in serums.[5]

Foot-and-mouth disease virus was propagated in tissue fragments suspended in a fluid medium first by Hecke[7] and later by Frenkel and Ribelin[6] and others. Virus produced by this method served as the antigen in the CF test. Recently, FMDV grown in cells cultured on glass has been employed as a complement-fixing antigen.[4] It is the purpose of this report to describe a method for the rapid production of material with high antigenicity in the CF test by growing FMDV in cells cultured on glass.

Materials and Methods

Virus.—Foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), that had been passed serially 100 times in outgrowths of bovine kidney cells was used in these studies. Harvested infectious fluids were adjusted to pH 7.5 and stored in ampules at −40 C. Before use, virus was thawed, clarified at 1,500 r.p.m. (380 x g), diluted 1:1 in growth medium containing 60% bovine serum (giving a final serum concentration of 30%), and warmed to 25 C.

Cultures.—Confluent sheets of cells were produced in 5-liter Povitsky bottles and 4-oz. prescription bottles from trypsin-dispersed calf kidney cells. Medium for cell growth was composed of Hanks' balanced salt solution, 2% bovine serum, and 0.5% lactalbumin hydrolysate. Penicillin G, dihydrostreptomycin sulfate, and phenol red were included in concentrations of 100 units/ml., 100 μg./ml., and 0.005%, respectively. Cell numbers were estimated from nuclei counts of cells treated with 0.1N citric acid and crystal violet.[12]

Infectivity Measurements.—The plaque method described by Bachrach *et al.*[1] was modified for this study. Virus was diluted decimally in culture medium to the desired dilution and 0.1-ml. aliquots from this dilution were transferred to two 4-oz. prescription bottle cultures of bovine kidney cells after the fluid had been aspirated from the cultures. The inoculum was distributed over the surface of the cells by tilting each bottle, and the cultures were incubated at 37 C. for 90 minutes. The inoculum was redistributed over the surface of the cells after 45 minutes of incubation. After 90 minutes of incubation, overlay was added consisting of Hanks' balanced salt solution, 10.0% bovine serum, 1.5% agar, and 1:30,000 neutral red. Cultures were incubated at 37 C. in an inverted position for 72 hours and the number of plaque-forming units (PFU) per milliliter was calculated for each sample.

Complement-Fixation Test.—Diluent.—Isotonic veronal buffered saline solution (VBS) was prepared as described by Mayer *et al.*[8]

Sheep Erythrocytes.—Sheep blood was obtained aseptically, diluted with an equal volume of Alsever's solution and stored at 4 to 8 C. for at least 4 days before use. When cells were needed, they were washed 3 times in VBS by centrifugation at 1,800 r.p.m. (600 x g). They were then diluted 1:45 in VBS and standardized in a spectrophotometer at a wave length of 550 mμ to give an optical density reading of 0.500.[12]

Hemolysin.—Commercial (Difco) hemolysin at its optimal dilution was used to sensitize the erythrocyte suspension as described by Osler *et al.*[10]

Complement.—Guinea pig serum that had been stored in a dry-ice chest was thawed and diluted on the day of the test.

Antiserum.—Susceptible adult guinea pigs were infected with FMDV-A119 by injecting intrader-

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Acknowledgment is made to Mr. Robert Goldsmith, Mr. Roger Williams, and Mr. Chester Finne for their technical assistance.

mally 0.2 ml. of a 1:10 suspension of infectious guinea pig metatarsal pad epithelium into each hind foot. After 30 days, each guinea pig was given 1 ml. of similar material intramuscularly. Ten days later, blood samples were obtained from these guinea pigs, and the blood was allowed to clot; the serum was removed, pooled, and stored in 5-ml. aliquots at −40 C. Serum testing 1:320 or greater against infected guinea pig pad vesicular fluid and epithelium was thawed, diluted 1:10 with VBS, and inactivated on the day of the test.

Complement-Fixation Test Protocols.—The reagents used were 0.25 ml. of a 1:10 serum dilution, 0.25 ml. of complement containing two 100% units, and 0.25 ml. of the appropriate dilution of antigen. Antigen in the fluid portion of each culture was diluted in twofold increments while the antigen obtained by fragmenting the cells was diluted from 1:100 to 1:300 at intervals of 25. The contents of each tube were mixed and incubated at 4 to 8 C. for 18 hours. After this fixation period, 0.5 ml. of optimally sensitized cells was added to each tube, mixed with the contents, and incubated at 37 C. for 30 minutes. The highest dilution of antigen that resulted in a 100% complement fixation was considered to be the antigen titer.

Besides the usual antigen, serum, and complement controls, fluid and fragmented cells from noninfected cultures (at similar recovery times as used for infected cultures) were included as antigen controls.

Production of Antigen.—Fluid was aspirated from Povitsky bottle cultures, each of which contained approximately 50 million cells. A 5-ml. inoculum of virus ($10^{7.7}$ PFU) was added to each culture and distributed over the cell surface. After 45 minutes of incubation at 37 C., 5 ml. of growth medium containing 30% bovine serum was added to each culture. The inoculum was redistributed over the cell sheet every 5 minutes during the 1st 45 minutes of incubation. The fluid was redistributed over the cell sheet every 15 minutes during the remainder of the incubation period.

After 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 29, and 36 hours of incubation, the cells and fluid from each of 2 cultures were harvested. The cells were scraped loose from the glass with a rubber policeman equipped with a long-handled, stainless steel rod. The cells and fluid were transferred to a chilled conical tube and centrifuged at 2,000 r.p.m. (680 x g) for 10 minutes in a holder containing chilled water. Supernatant fluid was aspirated and the cells were rinsed in chilled Hanks' balanced salt solution. Approximately 0.3 ml. of packed cells and 10 ml. of fluid were harvested from each culture. The cells were diluted 1:100 in Hanks' balanced salt solution and fragmented for 3 minutes in a chilled Omni mixer or micro Waring Blendor bowl. After centrifugation at 1,500 r.p.m. (380 x g) for 15 minutes to remove cellular debris, the fluid was assayed for virus infectivity and antigenicity and the results were adjusted to original cell volume. A sample of virus used for each virus inoculum was also assayed for virus infectivity and antigenicity.

Complement-fixing antigens were also produced in primary cultures of swine kidney cells and in cultures of a transmissible line of lamb testis cells by the methods described.

Results

The infectivity and the complement-fixing antigenicity of the virus in the fluid portion and in the fragmented cell portion of bovine kidney cultures at varying intervals after addition of virus to the cultures are presented (Table 1 and Fig. 1). These results are representative of 4 replications.

TABLE 1—Infectivity and Complement-Fixing (CF) Antigenicity of Foot-and-Mouth Disease Virus A119 in 5-Liter Povitsky Bottle Cultures*

Post-inoculation (hour)	Fluid portion		Cell portion	
	Log PFU/ml.	CF titer**	Log PFU/ml.	CF titer**
1	....	.....	....	1:100
4	8.4	1:40	9.6	1:175
6	8.7	1:80	9.8	1:275
8	8.7	1:160	9.6	1:275
10	8.8	1:160	9.4	1:200
12	8.5	1:160	9.2	1:200
14	8.4	1:160	9.1	1:200
16	8.3	1:160	8.9	1:175
18	8.4	1:160	9.1	1:200
20	8.2	1:160	8.6	1:175
22	8.2	1:160	8.3	1:175
24	7.8	1:160	8.4	1:150
29	....	1:160	....	1:100
36	....	1:160	....	1:100

* Each culture contained approximately 50 million cells covered by 10 ml. of fluid. Infectivity was recorded as number of plaque-forming units (PFU) per milliliter for each culture.

** Highest dilution of antigen giving complete complement fixation. Fluid diluted in twofold increments. Fragmented cell portion diluted 1:100 to 1:300 at intervals of 25. Virus infectivity and antigenicity titers adjusted to original cell volume.

Complement-fixing antigen was not detected in the inoculum. Complement-fixing antigen with a titer of 1:175 was obtained by fragmenting the cells as early as 4 hours after addition of virus to the cells. Two to 4 hours later, complement-fixing antigenicity reached its highest level in the cell portion of the cultures (1:275) and gradually declined during the remainder of the 36-hour incubation period. Even at 36 hours, however, the titer of the cell portion was high (1:100).

The fluid portion of the cultures exhibited complement-fixing antigenicity as early as 4 hours after addition of virus to the cells (1:40). Antigenicity reached a high level at 8 hours (1:160) and this level continued

during the remainder of the 36-hour incubation period.

Similar results were obtained when the virus was grown in primary swine kidney cells and in lamb testis cells that had been transferred through 40 passages.

Discussion

Methods have been described for the rapid production of material possessing high complement-fixing antigenicity by growth of FMDV in cells cultured on glass. These methods are based on: (1) achieving rapid attachment of virus to cells by applying the virus directly to the cells in a small volume and (2) allowing concentration of antigen to occur by limiting the volume of fluid covering the cells during liberation of antigen from the cells.

Complement-fixing antigen was not detectable in the virus inoculum. There was a reduction in the concentration of infectious virus in the inoculum due to (1) dilution of virus by the fluid that did not drain from the cells before addition of the inoculum, (2) thermal inactivation of the virus, and (3) attachment of virus to the cells. The removal of virus from the inoculum by the cells during attachment of virus resulted in the concentration of the virus in the cell portion of the culture. This adsorption of approximately 80% of the virus from 5 ml. of inoculum on approximately 0.3 ml. of cells was considerable. However, complement-fixing antigenicity in the cells at 4 hours (1:175) cannot be explained on the basis of adsorption alone since (1) the antigen titer at 3 hours was 1:100 in the cells when most of the virus from the inoculum had attached to the cells, (2) there was complement-fixing antigen in the fluid at 4 hours (1:40), and (3) there was an increase in complement-fixing antigen 2 hours later (6 hours) in both the fluid and cell portions of the culture. The rapid increase of complement-fixing antigen in bovine kidney cells cultured on glass and its accumulation in the fluid can be attributed to the rapid replication and liberation of the virus antigen.

Primary cultures of swine kidney cells and a transmissible line of lamb testis cells may also be employed for the rapid production of FMDV-A119 with high antigenicity.

De Boer and Bachrach [4] have described methods for producing FMDV-A119 complement-fixing antigen in bovine kidney cells cultured on glass. These authors were unable to detect complement-fixing activity in the fluid until 12 to 14 hours after infection of the cells. A maximum complement-fixing antigen titer of 1:16 was reached 8 hours or more after its appearance.

The method for rapid production of high concentrations of complement-fixing antigen in cells cultured on glass as described has application in the immunochemical studies of FMDV and perhaps of other viruses.

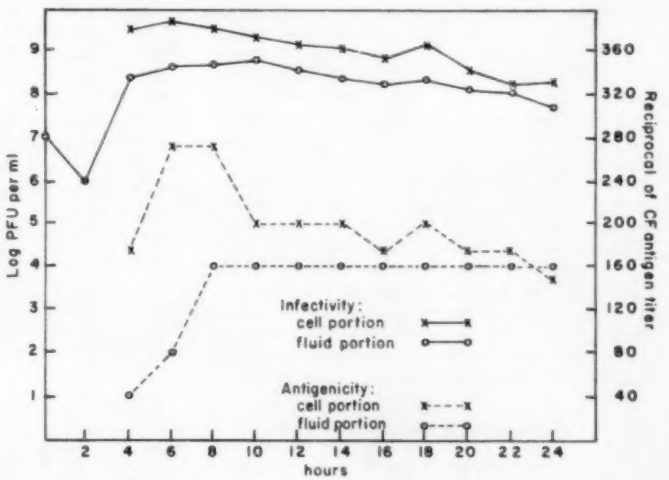

Fig. 1—Infectivity and complement-fixing antigenicity of fluid and cell portions of bovine kidney cell cultures inoculated with FMDV-A119. Each culture consisted of approximately 50 million cells covered by 10 ml. of fluid.

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SUMMARIO IN INTERLINGUA

Le Production de Virus de Febre Aphthose con Alte Antigenicitate a Fixation de Complemento

Esseva disveloppate un methodo pro le rapide production de virus de febre aphthose con alte antigenicitate a fixation de complemento in cellulas de ren bovin, de ren porcin, e de teste ovin culturate in vitro. Titros de antigeno de 1:275 esseva obtenite ab le portion cellular del culturas 6 horas post le addition de virus al cellulas. Un titro de 1:160 esseva obtenite ab le portion liquide 8 horas post le addition de virus al cellulas.

The Effect of Gaseous Ethylene Oxide on Dried Foot-and-Mouth Disease Virus

J. Tessler, M.S., and O. N. Fellowes, Ph.D.

SUMMARY

Dried foot-and-mouth disease virus (FMDV) was inactivated under definite minimal conditions of relative humidity and temperature when exposed to the action of gaseous ethylene oxide (ETO) mixed with trichloromonofluoromethane (TMM) and dichlorodifluoromethane (DDM). After FMDV was subjected to a 40% relative humidity (RH) for 30 minutes and then exposed to ETO at 98 or 78 F. and 40% RH for 5 hours, the virus was inactivated, as shown by tests in mice or tissue culture. Virus was not inactivated in 2 out of 3 trials after exposure to ETO for 5 hours at 40% RH and 98 F. without prior humidification. The gases TMM and DDM did not inactivate the dried virus in absence of ETO.

INTRODUCTION

The bactericidal properties of ethylene oxide (ETO) have been reported.[3,6] Virucidal activity of gaseous ethylene oxide on certain dried viruses has been studied by Mathews and Hofstad.[5] Foot-and-mouth disease virus (FMDV) in suspension was inactivated when exposed to ETO in the gaseous state [2,8] and in the liquid state.[9] Identical attempts to inactivate dried FMDV without adequate humidity control were not successful. Other investigators [3,4] had found that ETO was effective only in the presence of a certain amount of moisture. Similar observations were made in studies using bacteria.[4] In this study, a gas autoclave was modified to determine the action of ethylene oxide on dried FMDV under controlled conditions of humidity. An effort was made to find the minimal conditions which would result in complete inactivation as measured by mouse and tissue culture infectivity.

Materials and Methods

Virus.—Tissue culture fluid was pooled from primary cultures of bovine kidney epithelium which had been infected with FMDV, type A, strain 119 (A-119). The virus had undergone 87 consecutive passages in tissue culture before the pool was prepared. The harvested virus was placed in 15-ml. vials and stored at −40 C.

At the beginning of an experiment, 3 vials of virus were taken from the freezer, thawed, and pooled. Antibiotics were added at the concentration of 1,000 units of penicillin and 1 mg. of streptomycin for each milliliter of virus suspension. The virus material was evaporated to dryness by placing 10-ml. portions in each of 3 stainless steel pans (2 by 6½ by 11 inches) and keeping them for 18 hours at room temperature.

Gas Autoclave.—The gas autoclave was a standard steam sterilizer which had been modified by the American Sterilizer Company to simulate their combination autoclave "Cryotherm" units. The sterilizer contained an automatic heat control for the chamber and a manually set humidity control which supplied any desired relative humidity (RH) for experimentation. The autoclave was also equipped with a gauge which measured vacuum in inches of mercury and one which measured gas pressure in pounds per square inch. To conserve gas in the large sterilizing chamber, a rectangular space filler of stainless steel was placed in the chamber. In operation with the space filler, the chamber had a capacity of 546 liters of gaseous commercial ETO.*

Ethylene Oxide Mixture.—The ethylene oxide used in this study consisted of 11% of ethylene oxide and 89% of inert gases, trichloromonofluoromethane** (TMM) and dichlorodifluoromethane** (DDM). The final concentration of ETO in the autoclaves was calculated to be about 1,040 mg. per liter of space. All experiments were repeated, using different batches of commercial ETO.

Treatment Conditions.—A pan of dried virus was placed in the gas autoclave and the unit was evacuated to 27 inches of mercury. The vacuum was then maintained for 30 minutes under either normal chamber humidity (22% RH) or an induced

Received for publication Aug. 31, 1960.

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The authors thank R. Pease for technical assistance.

* Commercial ETO-Cryoxcide, manufactured by American Sterilizer Co., Erie, Pa.

** Freon 11 and Freon 12, respectively, manufactured by E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.

TABLE 1—Effect of Gaseous Ethylene Oxide* on Dried Foot-and-Mouth Disease Virus: Experimental Conditions in the Gas Autoclave

Experiment No.	Temperature (F.)	Relative humidity (%)	Exposure to relative humidity (hr.)	Exposure to ethylene oxide (ETO) (hr.)	Total time in autoclave (hr.)
1	78	22	5½	5	5½
2	78	40	5½	5	5½
3	98	40	5	5	5
4	98	40	5½	5	5½

* Ethylene Oxide-Cryoxcide, manufactured by the American Sterilizer Co., Erie, Pa., is a mixture of ethylene oxide, trichloromonofluoromethane, and dichlorodifluoromethane.

RH of 40%. The ETO was admitted in the form of 546 liters of gaseous commercial ETO. After 5 hours had elapsed, the chamber was evacuated to 26 inches of mercury. During this procedure, the gas was expelled through the laboratory bacterial-retentive filtering system. Atmospheric pressure was then restored to the chamber and the pan of virus was removed. The treated virus was reconstituted with 10 ml. of tissue culture (TC) fluid, composed of 2.0% bovine serum and 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution and adjusted to pH 7.0.

Test Conditions.—The procedures used in the 4 experiments of this study are outlined (Table 1). In the 1st experiment, the dried virus sample was exposed to ETO at 78 F. and 22% RH for a period of 5 hours following a 30-minute period at 22% RH under vacuum. In the 2nd experiment, the RH was increased to 40%; other conditions remained the same. The temperature was increased to 98 F. but the 30-minute prehumidifying period was omitted in the 3rd experiment. Finally, in the 4th experiment, the temperature was maintained at 98 F. and the ETO was admitted after 30 minutes. A relative humidity of 40% was maintained throughout this experiment.

Control Preparations.—Three different controls were used. One of these controls was FMDV which had been air-dried for 18 hours and then subjected to the experimental conditions of pressure, temperature, and humidity, but without the presence of ETO in the gas autoclave.

The 2nd control determined the loss in titer of FMDV which had been air-dried for 18 hours and held for 5½ additional hours at room temperature in the bacteriologic hood. The dried virus was reconstituted with 10 ml. of TC fluid at pH 7.0.

The 3rd virus control was the original fluid virus suspension assayed for titer in suckling mice and tissue culture.

Effect of Inert Gases.—The reaction of the mixture TMM and DDM without ETO on FMDV was tested. The equivalent mixture of inert gases was used as present in the ETO. A dried virus sample was exposed to the gases at 78 F. and 40% RH for a period of 5 hours following 30 minutes at 40% RH under vacuum. In addition, the 3 controls previously described were employed.

Infectivity Measurements.—Samples of the ETO-exposed virus controls were each prepared in serial dilutions in blanks of TC diluting fluid. Suckling mice, 7 to 9 days old, were used to determine titer of the samples which was calculated by method of Reed and Muench.[7] In addition, a plaque assay was performed as described by Bachrach *et al.*,[1] using primary cultures of bovine kidney epithelium in prescription bottles.

Results

Drying virus at room temperature resulted in a loss of approximately 1 log from initial titers ranging from $10^{7.9}$ to $10^{8.7}$ median lethal doses per milliliter as tested

TABLE 2—Effect of Gaseous Ethylene Oxide* on Dried Foot-and-Mouth Disease Virus: Infectivity Tests in Mice and Tissue Culture (TC)

Experiment No.	Titers**							
	Untreated		Air-dried for 18 hr.		Subjected to experimental conditions without ethylene oxide (ETO)		Virus experimentally exposed to ETO	
	Mice	TC	Mice	TC	Mice	TC	Mice	TC
1	8.7†	8.8	7.7	7.4	6.8	7.4	4.6	5.9
2	8.7	8.4	7.5	7.3	7.3	7.1	0.0	0.0
3	8.2	7.6	7.3	6.8	6.1	5.6	1.6‡	0.9‡
4	7.9	8.3	7.3	7.2	6.1	5.7	0.0	0.0

* Ethylene Oxide-Cryoxcide, manufactured by the American Sterilizer Co., Erie, Pa., is a mixture of ethylene oxide, trichloromonofluoromethane, and dichlorodifluoromethane. ** Titers obtained with mice are expressed as logarithms of LD_{50} of virus per milliliter; titers obtained with tissue culture are expressed as logarithms of plaque-forming units per milliliter. † Mean of 3 tests. ‡ In 2 out of 3 tests, infectivity was demonstrated; in only 1 test, no infectivity was shown.

in mice and from $10^{7.6}$ to $10^{8.8}$ plaque-forming units (PFU) per milliliter in tissue culture (Table 2). Gaseous TMM and DDM had no virucidal effect on dried FMDV (Table 3). In 3 separate experiments, the titer was reduced by about 2 logs when the dried virus was subjected to vacuum for 30 minutes at 78 F. with normal chamber humidity and then exposed to ETO 5 hours at the same humidity (experiment 1, Table 2). However, in 3 additional separate trials when samples of virus were placed under vacuum for 30 minutes at 78 F. and 40% RH, and then exposed to ETO for 5 hours at the same temperature and relative humidity, the FMDV was completely inactivated (experiment 2, Table 2).

TABLE 3—Effect of Trichloromonofluoromethane and Dichlorodifluoromethane on Dried Foot-and-Mouth Virus: Infectivity Tests in Mice and Tissue Culture (TC)

Virus controls							
Untreated		Air-dried for 18 hr.		Subjected to experimental conditions without trichloromonofluoromethane (TMM) and dichlorodifluoromethane (DDM)		Virus experimentally exposed to TMM and DDM	
Mice	TC	Mice	TC	Mice	TC	Mice	TC
8.0	8.3	7.8	7.3	7.2	7.0	7.7	7.3

Titers obtained with mice are expressed as logarithms of LD_{50} of virus per milliliter; titers obtained with tissue culture are expressed as logarithm of plaque-forming units per milliliter.

In 2 out of 3 separate experiments, after dried virus had been exposed to the same concentration of ETO for 5 hours at 98 F. and 40% RH without 30 minutes of prior humidification, infectivity was still present. Only in 1 experiment under the same conditions was no infectivity demonstrated. However, in 3 separate trials FMDV was completely inactivated when exposed to similar environment as that in experiment 3 with the addition of 30 minutes of prior humidification (experiment 4, Table 2).

No conditions of RH, temperature, and partial pressures of vacuum were capable of completely inactivating the virus in separate tests under these conditions.

Discussion

Many pieces of equipment and devices which have been exposed to pathogenic viral agents must be decontaminated or sterilized. Some of these devices would be harmed by a disinfecting process involving high temperatures or liquid chemicals. Therefore, virus sterilization by means of gas, such as ETO, would have many practical applications.

In most cases, the instruments and devices (electronic equipment, cameras, electric motors) would be contaminated with viruses that had become air-dried. Thus, the experiments described were designed to study inactivation of FMDV by ETO after virus material was desiccated. Apparently, virus retained a high level of infectivity as shown (Table 2) and could constitute a dangerous source of contamination.

The FMDV was rendered noninfectious as shown in experiment 2 (Table 2) when it first was placed under vacuum for 30 minutes at 40% RH and then exposed to ETO. These findings confirm reported observations of the effect of gaseous ETO on other viruses. The critical role of humidity was demonstrated by the fact that inactivation of FMDV by ETO did not occur below the 40% RH level. Kaye and Philips [3] reported a similar observation with bacterial spores.

Inactivation of dried FMDV on a nonporous surface showed that gaseous ETO was an adequate sterilizer when a preliminary period of rehydration was employed. The investigation reported here demonstrated that ethylene oxide was virucidal even under difficult conditions. Therefore, gaseous ETO could be considered a satisfactory decontaminant for dried FMDV adhering to surfaces.

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SUMMARIO IN INTERLINGUA

Le Effecto de Gasose Oxydo de Ethyleno Super Siccate Virus de Febre Aphthose

Siccate virus de febre aphthose esseva inactivate sub definite conditiones minimal de humiditate relative e de temperatura quando illo esseva exponite a gasose oxydo de ethyleno miscite con trichloromonofluoromethano e dichlorodifluoromethano. Post que le siccate virus de febre aphthose habeva essite subjicite a un humiditate relative de 40% durante 30 minutas, sequite per le exposition a oxydo de ethyleno a 98 o 78 F e 40% de humiditate relative durante 5 horas, illo esseva inactivate, secundo le evidentia de tests in muses e in histoculturas. Le virus non esseva inactivate in 2 de 3 essayos post le exposition a gasose oxydo de ethyleno durante 5 horas a 40% de humiditate e 98 F con le omission del previe humidification. Le gases trichloromonofluoromethano e dichlorodifluoromethano non inactivava le siccate virus de febre aphthose in le absentia de gasose oxydo de ethyleno.

Characterization of *Vibrio fetus* Antigens. IV. Study of Polysaccharide-Antibody Reactions by a Rapid Slide Gel Diffusion Technique

Miodrag Ristic, D.V.M., Ph.D., and D. K. Murty, B.V.Sc., M.S.

SUMMARY

A rapid slide gel diffusion technique has been developed for detection of antibodies against *Vibrio fetus*. By means of this technique, cross-precipitation reactions among 4 *V. fetus* polysaccharide antigens (HS) and 19 specific antiserums produced in rabbits were studied. The greatest degree of cross-reaction was revealed by antigen from isolate 3849, which reacted with 12 of the antiserums. A mixture consisting of equal quantities of antigens from isolates UF and 3849 reacted with antiserums to all 16 catalase-positive isolates. Antigen from isolate 3849 yielded precipitin lines with the serums of 12 cattle artificially infected with *V. fetus*. The specificity of the GP technique using this antigenic fraction was indicated by the absence of precipitin reactions with control samples of serum from *V. fetus*-free cattle and specific antiserums for *Leptospira* and *Brucella* species. Comparison of the serologic results obtained with the gel precipitation, hemolysis, and whole cell agglutination tests indicates that the latter two tests are inferior in accuracy to the gel precipitation test, and that the whole cell agglutination test is also less specific than the gel precipitation test.

INTRODUCTION

An immunologically active polysaccharide fraction, termed HS, was isolated from smooth *Vibrio fetus* organisms of bovine origin.[11] This fraction was employed for detection of *V. fetus* antibodies by inhibition of whole-cell agglutination,[11] by hemagglutination,[12] and by the hemolysis reaction.[13] In a preliminary study by Ristic and Brandly,[11] the HS substance was found to be serologically active when tested by Ouchterlony's plate gel diffusion method.[10]

The present report describes the analysis of antigenic reactions between polysaccharide fractions from 4 *Vibrio* isolates and 19 specific serum samples which were produced by rabbits inoculated with whole cell-saline suspensions of *V. fetus* isolates of diverse origin. The analysis was made by means of a modified rapid slide gel diffusion technique. In addition, the gel diffusion test was used to study the *V. fetus* antibody in serum from 12 artificially infected cows. A comparative study of the results of the gel diffusion test with those of other serologic procedures was made.

Review of Literature

A gel diffusion technique for detection of antigen-antibody reaction was originally described by Oudin.[8] In this technique, one of the reactants is allowed to diffuse through a vertical column of agar containing a uniform concentration of the 2nd reactant. Oakley and Fulthorpe[8] developed a modification of Oudin's technique in which both of the reactants are allowed to diffuse; this is known as the double-diffusion method. Ouchterlony[10] and Elek[2] described a double-diffusion technique carried out in a horizontal sheet of agar solidified in a Petri dish. Grasset *et al.*[3,4] introduced a rapid microtechnique for conducting this test, and Mansi[6] employed a slide gel diffusion method in the study of some viral and bacterial antigens. Dumbell and Nizamuddin[1] adopted the latter method in the study of smallpox virus. A modification of the Mansi[6] procedure by Murty,[7] designated as a rapid slide gel diffusion technique, was employed in the present study.

Materials and Methods

Medium.—The agar gel medium was prepared by admixing 0.5 Gm. of agar powder (Difco), 4.0 Gm. of sodium chloride, and 0.0005 Gm. of thimerosal* in 50 ml. of physiologic saline solu-

Received for publication Nov. 7, 1960.

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This investigation was supported in part by a research grant (E-3314) from the National Institute of Allergy and Infectious Diseases, USPHS.

* Merthiolate, product of Eli Lilly and Co., Indianapolis, Ind.

TABLE 1—The Origin and Biochemical Characteristics of Various Isolates of Vibrio fetus and Related Vibrio Strains

Isolate	Origin	Catalase production	H_2S production
3849	Bovine abortion	+	−
426		+	−
UF		+	−
B1		+	−
B4		+	−
B9		+	−
B10		+	−
B12		+	−
B14		+	−
B15		+	−
B16		+	−
F		+	−
55	Bovine nonabortion	−	+
DL		−	+
III	Ovine abortion	+	−
K		+	−
6867	Ovine nonabortion	−	+
1134	Human Idiopathic infection	+	−
1133		+	−

tion buffered with 0.15M of phosphate. More recent studies have shown that 0.43 Gm. of sodium chloride per 50 ml. of physiologic saline solution will produce similar results. The final pH of this mixture was 7.2. The mixture was sterilized by autoclaving at 120 C. for 15 minutes and was maintained in a liquid state (60 C.) until poured on the slides.

Slides.—A rectangular area, 30 x 25 mm., was marked on a piece of paper, and the pattern of cups for antigen and serum was drawn in its middle. Various patterns consisting of 3, 4, 5, 7, or 9 cups were made, depending upon the requirements of the study. Most frequently, however, a 5-cup pattern was used which consisted of 1 central reservoir and 4 peripheral cups, each 4 mm. in diameter, placed 2.5 mm. from the central cup (Fig. 1). A clean slide was placed over the paper pattern and 2 horizontal lines, 3 cm. apart, were drawn on the slide with a wax pencil, leaving a 2-cm. area free on each end (Fig. 1). A layer of liquid agar was placed on this area by allowing

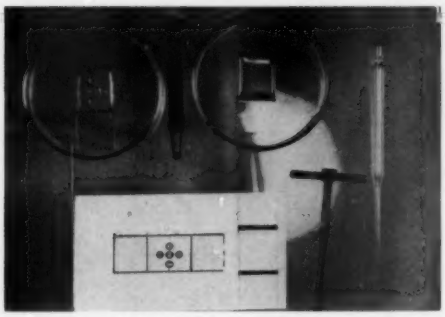

Fig. 1—Illustration of the equipment needed for conducting the rapid slide gel diffusion technique.

1.5 ml. to flow onto it gently from a graduated pipette. After the agar was solidified, the slide was placed in a Petri dish and kept in the refrigerator for approximately 2 hours before the reservoirs were cut. The slide was then placed over the paper having the selected pattern, and the cups

TABLE 2—Results of Gel Precipitation, Whole-Cell Agglutination, and Hemolysis Reactions Between Antigens Derived from 2 Vibrio fetus Isolates (UF and 3849) and Antiserums Against Smooth Forms of 19 Vibrio fetus Isolates

	Antigen*						
	UF			3849			UF + 3849
Antiserum	AG titer	HL titer	GP reaction	AG titer	HL titer	GP reaction	GP reaction
F	2,520**	10,240	+	1,280	0	−	+
UF	5,120	40,960	+	160	0	+	−
3849	640	0	−	5,120	20,480	+	+
B1	0	0	−	320	640	+	+
B4	0	160	−	640	2,560	+	+
B9	0	0	−	40	2,560	+	+
B10	640	5,120	+	160	160	−	+
B12	0	0	−	10,240	5,120	+	+
B14	0	0	−	320	5,120	+	+
B15	20	640	−	1,280	5,120	+	+
B16	40	640	−	320	5,120	+	+
426	0	640	−	160	5,120	+	+
55	0	0	−	0	0	−	−
DL	0	160	−	0	160	−	−
III	5,120	40,960	+	0	0	−	+
K	5,120	20,480	+	0	0	−	+
6867	0	0	−	0	0	−	−
1134	40	40	+	40	40	+	+
1133	0	0	+		80	+	+

GP = gel precipitation; AG = whole-cell agglutination; HL = hemolysis; + = precipitin line present; and − = precipitin line absent.

* Polysaccharide fraction HS. ** Titer expressed as reciprocal of serum dilution.

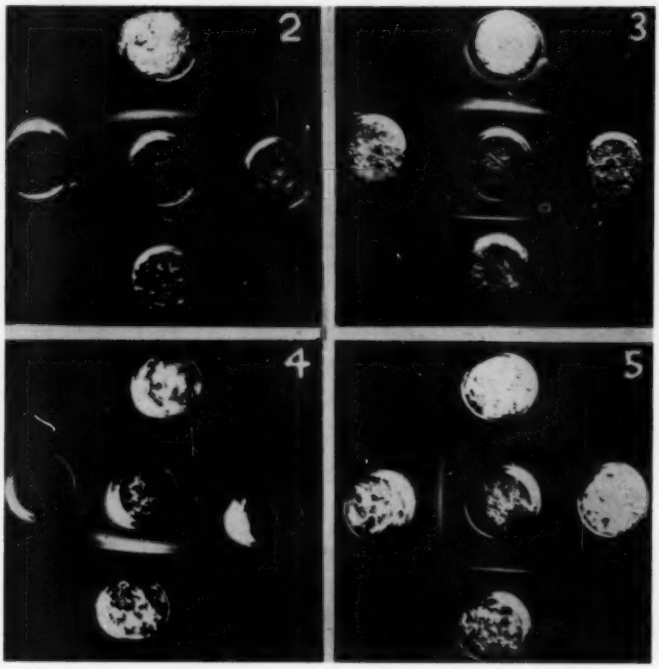

Fig. 2-5—Precipitin reaction in gel between antigen 3849 (middle cup) and 1, 2, 3, and 4 positive serums (Fig. 2, 3, 4, and 5, respectively).

TABLE 3—Cross Precipitation Reactions in Gel of HS Antigens Derived from 4 Vibrio fetus Isolates and Antiserums Against Smooth and Rough Variants of 7 Vibrio fetus Isolates

Antiserum	Antigens				
	3849	UF	B14	B10	3849 + UF
B1 (S)*	+	−	−	−	+
B1 (R)**	+	+	−	+	+
B4 (S)	+	−	−	−	+
B4 (R)	+	+	−	+	+
B10 (S)	−	+	−	+	+
B10 (R)	+	+	−	+	+
FS	−	+	−	+	+
FR	+	+	−	+	+
UFS	+	+	+	+	+
UFR	−	+	−	+	+
III (S)	+	+	+	+	+
III (R)	−	+	−	+	+
B15 (S)	+	−	−	−	+
B15 (R)	+	+	+	+	+

+ = Precipitin line present; − = precipitin line absent.
* (S) = Antiserum against smooth variant.
** (R) = Antiserum against rough variant.

were cut in the medium by placing the slide over the paper pattern and cutting holes with a metal cork borer of the proper diameter. The medium was removed from the cups by suction with a Pasteur pipette (Fig. 1).

The cups were filled with the reactants by means of finely drawn Pasteur pipettes, and the slides were incubated at approximately 22 to 24 C. Usually, antigen was placed in the central cup and antiserums in the peripheral ones. During the 1st several hours of the test, the cups were refilled 3 or 4 times at intervals of an hour. Refilling of the cups increased the intensity of the precipitin lines and shortened the time necessary for their appearance. Precipitin lines usually became visible between the 12th and 18th hour of the test and were distinct by 24 to 48 hours. The test was terminated at 72 hours. Precipitin lines were viewed by means of indirect light. When desired, specimens could be preserved at 4 C. for 2 to 3 weeks.

TABLE 4—Results of Gel Precipitation (GP) Reactions Between the HS Fractions from Vibrio fetus Isolate UF and Antiserums Prepared Against Smooth and Rough Variants of 7 Vibrio fetus Isolates

Antiserum	Variant	GP reaction
UP	Smooth	+ 2 lines
	Rough	+ 1 line
B6	Smooth	−
	Rough	+ 1 line
B10	Smooth	+ 2 lines
	Rough	+ 1 line
F	Smooth	+ 2 lines
	Rough	+ 1 line
B4	Smooth	+ 2 lines
	Rough	+ 1 line
B1	Smooth	−
	Rough	+ 1 line
III	Smooth	+ 2 lines
	Rough	+ 1 line

+ = precipitin line present; − = precipitin line absent.

TABLE 5—Gel Precipitation, Whole-Cell Agglutination, and Hemolytic Reactions of Serum from Cows Bred to a Vibrio fetus-Infected Bull

Cow No.	Date bred to *V. fetus*-infected bull	Date *V. fetus* isolated	Serologic Reactions																	
			7/7			7/14			7/21			8/3			8/18			9/11			9/24			10/6			10/20		
			AG*	HL†	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP	AG	HL	GP
4139	6/24/59	7/ 8/59	+	−	+	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4154	6/17/59	6/12/59	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	−	+	−	+	+
4251	5/31/59	6/12/59	−	−	+	−	−	+	+	+	+	+	−	+	+	−	+	+	+	+	−	+	+	+	−	+	−	+	+
4276	6/25/59	7/ 8/59	−	−	+	−	−	+	+	−	+	+	+	+	−	−	+	+	+	+	−	−	+	+	−	+	−	+	+
4260	7/ 5/59	No *V. fetus* isolated	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Date bred to *V. fetus*-free bull																												
4275	6/14/57	No *V. fetus* isolated	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4278	6/12/59	No *V. fetus* isolated	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−

AG = whole cell agglutination test; HL = hemolytic test; GP = gel precipitin test. Whole *V. fetus* cells of isolate 3849 were used in the AG test and the HS fraction derived from this isolate was used in GP and HL tests.

* Titers of 1:60 and higher have been considered as positive reaction. † Titers of 1:80 and higher have been considered as positive reaction.

The whole-cell agglutination and the hemolytic tests were carried out according to the procedures already described.[12, 13]

Antigens.—The origin and the biochemical characteristics of the *V. fetus* and the related *Vibrio* isolates used are shown (Table 1). The HS polysaccharide fractions were prepared from cultures of isolates 3849, UF, B14, and B10. A mixture of HS fractions derived from the UF and 3849 isolates was also prepared from equal quantities of the 2 antigens. The antigen concentration was 5 mg. per milliliter of physiologic NaCl solution.

Antiserums.—Whole *V. fetus* cells derived from selected smooth and rough variants were used for production of the specific antiserums studied. These antiserums were produced in hyperimmunizing rabbits in accordance with the procedure described by Ristic and Brandly.[11] Serum samples from 12 artificially infected cows, 7 of which had previously been vaccinated with *V. fetus* bacterin according to the protocol shown (Table 6), were also studied. These serum samples were received from Dr. A. H. Frank.* The present study was confined to the serologic evaluation of these serum samples; the protective value of the vaccines was not determined.

Results

The results are shown (Table 2) for gel precipitation (GP) of HS fractions; whole-cell agglutination (AG); and hemolysis (HL) reactions between *V. fetus* antigens (derived from isolates UF and 3849 and a mixture of these 2) and specific serums against smooth variants of 19 *Vibrio* isolates.

On the basis of the GP test, antigen from isolate UF reacted with 7 antiserums, and antigen from isolate 3849 reacted with 12 (Fig. 2–5). Antigen from isolate UF reacted with 9 serums in the AG test and with 11 serums in the HL test. Antigen from isolate 3849 reacted with 13 serum samples in both the AG and HL tests. Reactions between antigen from isolate 3849 and antiserum against isolate UF occurred in the GP and AG tests, while antigen from isolate UF reacted with serum of isolate 3849 in the AG test only. When equal quantities of antigen from the UF and 3849 isolates were mixed and used in the GP test, reactions occurred with the serum samples of all 16 catalase-positive *V. fetus* isolates.

Cross serologic reactions between the HS antigens from the 4 isolates and the antiserums against smooth and rough variants of 7 *V. fetus* isolates are indicated (Table 3). Antigen B14 reacted with 3 of the

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serum samples. The remaining 3 antigens reacted with the antiserums of at least the smooth or rough variant of each of 7 isolates. A greater degree of cross reactivity occurred with the antiserums against the rough variants than against the smooth. The combination of antigens 3849 and UF reacted with antiserums to all 14 variants.

The results of the precipitation reactions between the HS fraction of isolate UF and antiserums against the smooth and rough variants of the homologous and of 6 heterologous *V. fetus* isolates are shown (Table 4). Antiserums for antigens from the smooth variants of strains B6 and B1 did not react with the HS fraction from isolate UF. All the other serums tested formed precipitin lines in gel with the HS fraction from isolate UF. Two precipitin lines were formed with the smooth variant, and 1 precipitin line was formed with the rough variant.

The AG, HL, and GP reactions of serums from cows bred to a bull infected with *V. fetus* are shown (Table 5). With the exception of cow 4260, whose serum reacted positively in the GP test at only 3 testing periods, the serum samples of the remaining 4 animals, all of which had been bred to an infected bull, reacted in all 9 tests conducted during a period of approximately 3 months after infection. These serum samples reacted in the HL and AG tests during part, but not all, of the testing periods. The serums of 2 *V. fetus*-free cows reacted in the AG test in 7 instances, but were negative in GP and HL tests.

Table 6 shows that after vaccination each of 8 cows was found to react to the GP test for approximately 3 months. During the 4th month, reactions were not observed regularly. The serum of cow 4431 produced a faintly visible precipitin line during 2 testing periods prior to vaccination and, by the 3rd prevaccination testing period, produced a clearly visible precipitin line. The remaining animals, regardless of the type of bacterin used, produced the 1st serum precipitin response in the earliest post-vaccination test conducted, which was 11 days after vaccination.

Specific rabbit antiserums prepared against *Brucella abortus* and 4 leptospiral strains *(L. canicola, L. icterohaemorrhagiae, L. pomona,* and *L. sejroe)* did not react in the GP test with *V. fetus* antigens of isolates 3849, UF, B14, and B10.

TABLE 6—Serum Precipitin Response in Cows Vaccinated Against Vibriosis and Subsequently Infected

Animal No.	Date vaccinated	Date bred to infected bull	Date *V. fetus* isolated	Date blood samples obtained														
				6/9	7/6	7/14	7/28	8/4	8/10	8/17	8/25	8/31	9/10	9/15	9/22	9/30	10/6	10/13	10/20	10/27
Live-Virus Vaccine																				
4412	7/17	9/14	9/22	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
4417	7/17	9/14	9/22	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
4428	7/17	9/6	9/10	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4457	7/17	10/10	10/22	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Killed-Virus Vaccine																				
4405	7/17	10/18	10/22	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	±
4415	7/17	9/4	10/7	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
4431	7/17	Not bred	No *V. fetus* isolated	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
4447	7/17	9/4	9/22	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−

+ = Clearly visible precipitin line; ± = faintly visible precipitin line; − = no precipitin line. Live = saline suspension of live *V. fetus* cells; killed = saline suspension of *V. fetus* cells killed in 0.3% formaldehyde-saline solution.

The density of the bacterin approximated the density of tube 3 of McFarland's nephelometer. Ten milliliters of the vaccine were administered subcutaneously. The serum samples were provided by Dr. A. H. Frank, USDA, Beltsville, Md.

Discussion

The lack of an accurate and practical diagnostic technique had been a major obstacle in devising an effective control and eradication program for bovine vibriosis. It is generally agreed that detection of blood serum agglutinins by the whole-cell tube agglutination test is not a reliable diagnostic procedure. The antigen employed is apparently not satisfactory. The major difficulties in selecting and maintaining a dependable and stable whole-cell *V. fetus* antigen lie in (1) antigenic heterogeneity among different isolates of *V. fetus* and (2) relatively rapid dissociation of *V. fetus* cultures *in vitro* from the originally isolated smooth form to several spontaneously agglutinating nonsmooth forms. The cervico-vaginal mucus agglutination test is apparently a more specific diagnostic procedure, but it is not practical and has limited use for diagnosis of *V. fetus* infections.

The GP test here described appears to be both practical and accurate in diagnosing *V. fetus* infections. Except for a minor discrepancy in the case of cow 4431, the GP test revealed promptly and clearly the reactions which were anticipated from the history of the animals studied. On the contrary, the whole-cell agglutination test failed in that it was positive with serum samples from 2 *V. fetus*-free cows. The difference in reactivity between the HS antigen and the whole-cell antigen results primarily from the difference in their nature. The apparent advantage of the HS antigen over the whole-cell antigen lies in its uniformity and greater stability, and also in the accuracy with which it may be standardized.

The serologic reaction based on the antigen and antibody partition in the aqueous phase of a gel is generally recognized as a delicate and highly specific procedure. The method is accepted as most dependable for the analysis of antigen-antibody systems. Because of this, the GP technique described here would appear to be convenient and accurate for studying the complex antigenic character of *V. fetus* isolates, including those of bovine, human, and ovine origin. The position and curvature of the precipitin line, with reference to the antigen and antibody reservoirs, is influenced by the molecular size of the reactants, their diffusion coefficients, their concentrations, the conditions of the environment, and the composition of the gel medium. Thus, the GP technique offers a simple tool for making approximate estimates of the molecular sizes of antigens and antibody.[5]

Precipitin tests are in general not well suited for titration of antibody; thus, the GP test will be of comparatively little use for serum titer determinations. However, the test reveals a clear difference between negative and positive serum, and thus makes the determination of the titer less important. Nevertheless, it can be expected that information concerning serum titers may prove necessary to complete our knowledge of the complex mechanism of *V. fetus* infections.

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SUMMARIO IN INTERLINGUA

Characterisation de Antigeno *Vibrio fetus*. IV. Studio de Reactiones de Polysaccharido con Anticorpore per Medio de un Rapide Technica de Diffusion in Gel sur Lamina Porta-Objectivo

Esseva disveloppate un rapide technica per diffusion in gel a lamina porta-objectivo pro le detection de anticorpore anti *Vibrio fetus*. Per medio de iste technica, reactiones de precipitation cruciate inter 4 antigenos polysaccharidic *V. fetus* e 19 specific antiseros producite in conilios esseva studiate. Le plus alte grado de reaction cruciate esseva revelate per antigeno ab le isolato 3849 le qual reageva con 12 del antiseros. Un mixtura consistente de quantitates equal de antigenos ab le isolatos UF e 3849 reageva con antiseros a omne le 16 isolatos positive pro catalase. Antigeno ab isolato 3849 produceva lineas de precipitina con le seros de 12 pecias de bestial que habeva essite artificialmente inficite con *V. fetus*. Le specificitate del technica de precipitation in gel, utilisante iste fraction antigenic, esseva indicate per le absentia de reactiones precipitinic con specimens de controlo de sero ab bestial libere de *V. fetus* e de specific antiseros anti species de *Leptospira* e *Brucella*. Le comparation del resultatos serologic obtenite per le tests de (1) precipitation in gel, (2) hemolyse, e (3) agglutination de cellulas integre indica que le secunde e le tertie de iste tres es inferior in accuratia al test de precipitation in gel e que le tertie (i.e. le test de agglutination de cellulas integre) es, in plus, minus specific que le test de precipitation in gel.

Studies in Anaplasmosis. IV. Development of the Causative Agent in Deer Erythrocytes Transfused into Calves

Julius P. Kreier, V.M.D., M.S., and Miodrag Ristic, D.V.M., Ph.D.

SUMMARY

Blood from *Anaplasma*-free deer was introduced into 2 calves with acute *Anaplasma marginale* infections. By both histochemical and immunofluorescent staining techniques it was demonstrated that the *Anaplasma* organisms entered and developed in the newly introduced mature deer erythrocytes.

INTRODUCTION

The role of the marginal *Anaplasma* body in the etiology of the disease anaplasmosis is still obscure. Recent immunofluorescent studies,[7,8] in which direct and indirect specific staining of *Anaplasma* with labeled antibody was demonstrated, offer strong support for the assertion that the marginal body actually stimulates the production of the antibody. Electron microscopic studies of erythrocytes of cattle with anaplasmosis have shown that the marginal *Anaplasma* body is similar structurally to the inclusion bodies produced by some members of the psittacosis-lymphogranuloma group of organisms. The inclusion body is composed of 2 to 8 smaller units, called initial bodies, which have morphologic characteristics similar to those of the rickettsiae.[2,4,5] Preliminary electron microscopic studies by Ristic and Watrach [5] have shown that the initial bodies reproduce in mature erythrocytes by means of binary fission. On the basis of this static evidence, these workers advanced a hypothesis that the initial *Anaplasma* body is capable of growing, multiplying, and forming marginal *Anaplasma* bodies in mature erythrocytes. However, evidence other than that from electron microscopic studies to prove that the marginal body can develop in the adult red blood cell has not been produced. The complete lack of success of all attempts to grow the *Anaplasma* organisms in red blood cells *in vitro* has caused some workers to suggest that the actual site of *Anaplasma* growth may be the bone marrow, and that the appearance of the organism in the erythrocytes is merely a retarded manifestation of active growth.

This report shows, by means of histochemical and immunofluorescent techniques, that *Anaplasma* can enter fully developed deer erythrocytes after they have been transfused into *Anaplasma*-infected calves.

Materials and Methods

Two Virginia white-tailed deer *(Dama virginiana)*, approximately 1 year old, and 2 Holstein-Friesian calves, 6 to 9 months of age, were used in this study. The source of the *Anaplasma* was blood from Florida cattle acutely affected with anaplasmosis. The organisms were maintained in a carrier cow. The experimental calves and deer were splenectomized at least 2 months before the start of the experiment. During the 2-month pre-experimental period, the animals were examined 3 times a week. The animals had no evidence of anaplasmosis on examination of blood films stained by the Wright-Giemsa technique, had normal hematocrit values, and had no demonstrable anti-

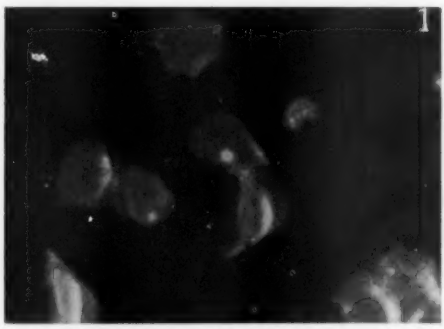

Fig. 1—Anaplasma marginale in deer erythrocytes. The blood film was stained with fluorescein-labeled, anti-Anaplasma antibody of bovine origin. This figure illustrates the characteristic shape of deer erythrocytes, and these may be compared to the deer erythrocytes shown in Figure 7.

Received for publication Jan. 24, 1961.

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This investigation was supported in part by a research grant (E-3315) from the National Institute of Allergy and Infectious Diseases, USPHS.

bodies in anaplasmosis complement-fixation tests. Animals were infected by intravenous inoculations of 100 ml. of fresh blood from the carrier cow.

Blood compatibility was tested by tube agglutination tests, using a 2% suspension of washed deer erythrocytes and fresh calf serum. Transfusion was not attempted if fresh undiluted calf serum agglutinated the erythrocytes of the potential donor deer. Two successful transfusions of blood were made from noninfected deer to acutely infected calves in which approximately 40% of their erythrocytes harbored *Anaplasma marginale*. One calf (47) was given 1.0 liter of deer blood and the other (1696) was given 1.5 liters. Blood for transfusions was collected in 500-ml. vacuum flasks containing A.C.D. solution.* Each 100 ml. of this solution contains: 0.44 Gm. of citric acid, 1.32 Gm. of sodium citrate, and 1.47 Gm. of dextrose. Each 120 ml. of solution will prevent coagulation of 480 ml. of blood. The equipment used for the transfusions was a disposable unit such as is used for transfusions in human patients.

The blood was transfused into the jugular vein. During the transfusions, the calves were tied on a table. The rate of transfusion was reduced if the rate of the heart beat increased, or if respiratory distress developed. Other than accelerated heart beat and respiratory distress, signs which were controlled by slowing or temporarily stopping the transfusion, no gross transfusion reactions occurred in either calf. About 4 hours were required to complete the transfusions in each calf. The deer blood was collected from the jugular vein of the donor animal immediately before the start of the transfusion.

* Baxter Laboratories, Inc., Morton Grove, Ill.

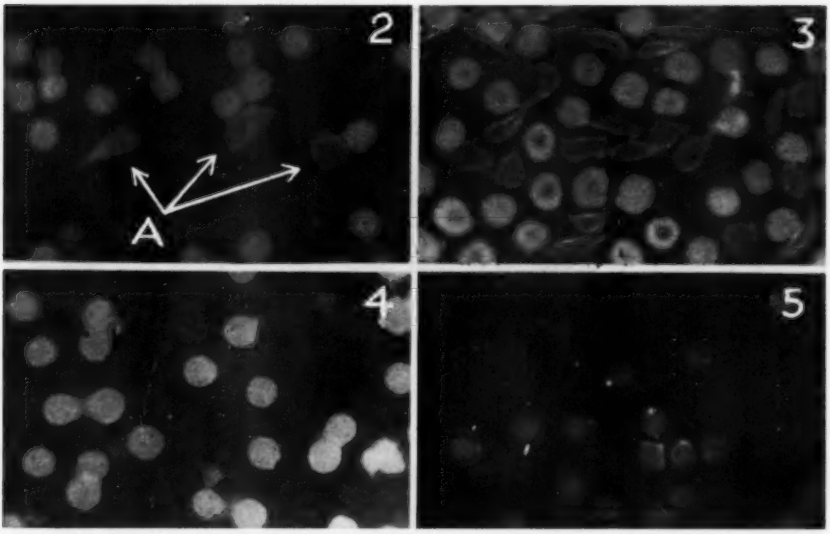

Fig. 2—Deer erythrocytes in bovine circulation; slide made from blood collected immediately after completion of transfusion of normal deer blood into an acutely infected calf (47). The blood film was treated with fluorescein-labeled, anti-bovine erythrocyte serum and fluorescein-labeled, anti-Anaplasma serum. Notice the 3 (arrow A) nonfluorescing deer erythrocytes and many fluorescing bovine erythrocytes.

Fig. 3—Artificial mixture of deer and bovine erythrocytes. Slide treated with anti-bovine fluorescein-labeled antiserum. Notice strongly fluorescent bovine erythrocytes. Deer erythrocytes are also identifiable on the basis of their lack of fluorescence and somewhat characteristic morphology.

Fig. 4—Blood film made from blood of a calf (47) just before transfusion and stained with fluorescein-labeled, anti-bovine erythrocyte antiserum and fluorescein-labeled, anti-Anaplasma anti-serum. The erythrocytes appear as intensely fluorescing, uniform, round structures. Notice absence of sickle and non-fluorescent cells. The Anaplasma bodies present are not visible because the fluorescence of the Anaplasma is masked by the fluorescence caused by the anti-erythrocyte serum.

Fig. 5—Anaplasma marginale infected bovine blood film preparation treated with bovine origin, anti-Anaplasma labeled antibody. This slide was prepared from blood drawn from an experimental calf (1696) just before transfusion.

TABLE 1—Concentration (%) of Deer Erythrocytes and Incidence of Their Infection with Anaplasm marginale in Calves Transfused with Deer Blood

Calf No.	Pretransfusion		Post-transfusion		Hours after transfusion															
					2		4		6		8		10		12		16		24		40		48	
	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.
47	0	....	2–5*	—	2–5	—	2–5	—	2–5	—	2–5	—	1–2	+	0–1	+	0–1	+	0	—	0	—	0	—
1696	0	....	3–6	—	2–5	—	2–5	—	2–5	—	2–5	+	1–3	+	1–2	+	1–2	+	0–1	+	0	—	0	—

E = erythrocytes; A = *Anaplasma*.

* The distribution of deer erythrocytes in the blood films made from the samples collected was very uneven. Frequently deer erythrocytes tended to cluster in certain areas of the slide. The figures given are upper and lower limits in fields observed. Marginal *Anaplasma* bodies are noted as observed (+) or not observed (—) in these deer erythrocytes. The percentage of infected deer erythrocytes was low and did not exceed 2%.

Samples of blood from the recipient animal were collected in a tube containing heparin (10 units of sodium heparin in 2 ml. of blood) before the transfusion was started as well as immediately after it was completed, and then every 2 hours for the next 48 hours. Samples were also taken from the transfusion bottle just before transfusion began. Films were made from these blood samples for staining by a combination of the Wright and Giemsa techniques and by the fluorescein-labeled antibody technique. For the Wright-Giemsa staining, thin blood films were fixed in absolute methyl alcohol for 3 minutes, dried, and flooded with Wright's stain for 2 minutes. An equal volume of Sorenson buffer at pH 6.8 was then added to the Wright's stain on the slide. The mixture was allowed to stand on the slide for 4 minutes, and the slide was washed and covered with freshly made Giemsa stain (3 ml. of concentrated stain to 50 ml. of Sorenson phosphate buffer, pH 6.8) for 30 minutes. Finally, the slide was washed and dried. The fluorescein-labeled antiserums were prepared and the slides were stained according to the procedures described by Ristic *et al.*,[7,8] except that commercially prepared fluorescein isothiocyanate was used.* The antiserums from which immune *Anaplasma* globulin was secured were obtained from experimentally infected calves at the height of infection. The anti-bovine erythrocyte antiserum was produced in rabbits. It was necessary to absorb the rabbit anti-bovine serum and the bovine anti-*Anaplasma* serum with deer cells in order to remove anti-deer hemagglutinins. For the fluorescent studies, thin blood films were fixed in absolute alcohol for 15 seconds and air dried. A circle about 0.5 inch in diameter was drawn on the slide with a lead pencil. Several drops of labeled anti-bovine erythrocyte serum were placed on the slides and spread to cover the circled area. The slides were placed in Petri dishes containing moist paper and incubated for 1 hour. They were then washed for 10 minutes in phosphate-buffered saline solution at pH 7.2 and in distilled water for 5 minutes and air-dried. Labeled anti-*Anaplasma* serum was placed over the same circled area and specimens were incubated, washed, and dried as described above. A cover slip was mounted on each slide with buffered glycerol (pH 7.2). The cover slip was fastened to the slide by painting its edges with clear fingernail polish. The mounted slide was then examined with an ultraviolet microscope.

Results

Marginal *Anaplasma* bodies in deer and bovine erythrocytes were identifiable in blood films treated with fluorescein-labeled, anti-*Anaplasma* antibody as bright yellow-green, fluorescing, round bodies within the relatively nonfluorescing red blood cells (Fig. 1, 5). Deer erythrocytes were detec-

* Nutritional Biochemicals Co., Cleveland, Ohio.

table by their lack of fluorescence in blood films containing artificially admixed deer and bovine erythrocytes that had been treated with fluorescein-labeled, anti-bovine erythrocyte serum (Fig. 3). The spindle- or sickle-shape of many deer cells also aided in their identification.

Two calves with acute anaplasmosis tolerated the introduction of 1.0 liter (47) and 1.5 liters (1696) of noninfected deer blood (Table 1). Films made from the blood of these infected calves before the transfusion, treated with fluorescein-labeled anti-bovine erythrocyte serum, had a uniform distribution of fluorescing, nonsickled, cells (Fig. 4). Red blood cells from the donor deer contained no bodies which stained like *Anaplasma* bodies with the Wright-Giemsa or fluorescent techniques. Blood collected from the calves immediately after the completion of the transfusions, and every 2 hours thereafter for 6 hours, contained 2 to 5% deer erythrocytes; no marginal bodies were observed in these cells (Fig. 2). At 8 and 10 hours after the completion of the transfusion, characteristic marginal bodies were found in the deer cells in both the fluorescein-labeled antibody and Wright-Giemsa stained blood films (Fig. 6–8). Approximately 1 to 2% of the deer erythrocytes present in the bovine blood circulation after transfusion developed marginal *Anaplasma* bodies. The marginal bodies in the deer erythrocytes varied from 0.4 to 1.5 μ in diameter. From the 14th to the 24th hour after transfusion, 1% or less of the deer erythrocytes could be observed. Thereafter and until 48 hours after transfusion, at

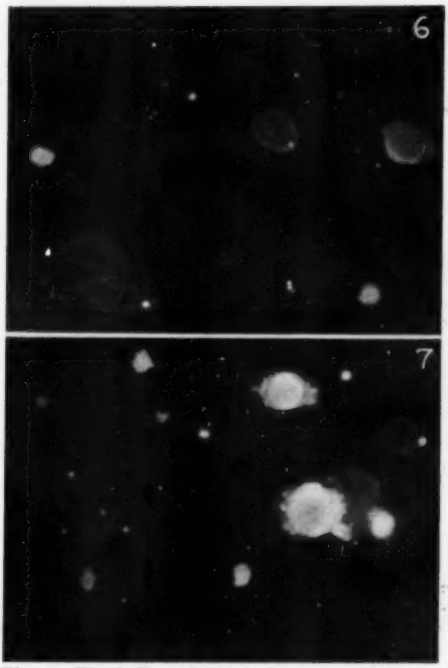

Fig. 6—Blood film of blood from an acutely infected calf (1696) 8 hours after the calf had been given a transfusion of normal deer blood. The slide was treated with fluorescein-labeled, anti-bovine red blood cell serum and fluorescein-labeled anti-Anaplasma serum. Notice non-fluorescent deer erythrocytes, 2 of which contain highly fluorescent marginal Anaplasma bodies. Fluorescent bovine erythrocytes are also present.

Fig. 7—This figure is comparable to Figure 6, with the exception that the blood was obtained from a calf (1696) 10 hours after transfusion.

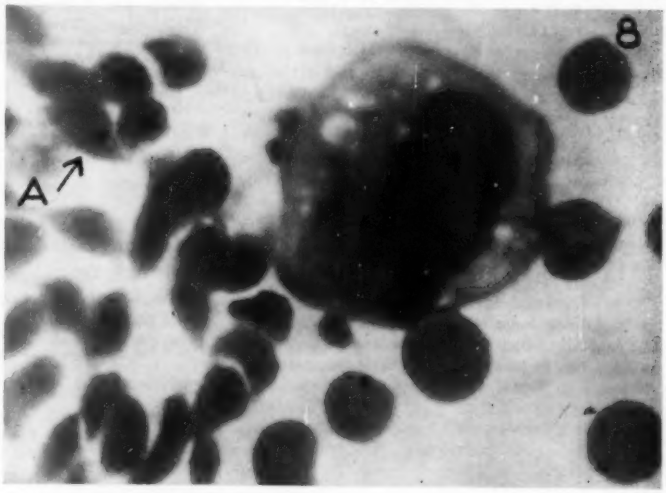

Fig. 8—Wright-Giemsa stained blood film made from blood taken from an infected calf (1696) 10 hours after the calf was given a transfusion of normal deer blood. Notice the sickle-shaped deer erythrocytes, 1 of which contains a marginal Anaplasma body (arrow A). Several bovine erythrocytes containing Anaplasma bodies are also visible.

which time observations were discontinued, deer erythrocytes were not observed in any of the calf blood specimens.

Discussion

This experiment demonstrated the development of marginal bodies in adult erythrocytes. Infection was shown directly; *i.e.*, by introducing *Anaplasma*-free deer erythrocytes into infected calves, and then demonstrating the marginal *Anaplasma* bodies in these cells. Development of the organism in these deer erythrocytes was substantiated by the appearance of marginal bodies of different sizes. This work, therefore, confirms the hypothesis of growth and development of the adult erythrocyte, which Ristic [4] and Ristic and Watrach [5] advanced as a result of their electron microscopic studies. The latter studies showed that the *Anaplasma* initial body may occur singly or in 2's, 3's, etc., as well as grouped into complete marginal bodies. This finding indicated that the initial body multiplies in the mature erythrocyte, probably by means of binary fission, to form the marginal body.

The experimental work reported in this paper demonstrates that the marginal body can develop in the mature erythrocyte. The means or mechanism of passage of the organism from erythrocyte to erythrocyte is still not clear, nor is the existence of intermediary cells in the cycle to be precluded. Recent work by Ristic and Watrach,[6] which has demonstrated the occurrence of *A. marginale* in bovine platelets, suggests that the platelets may be involved in the cycle, possibly as a vehicle of transport from erythrocyte to erythrocyte.

The time which elapsed between the introduction of the deer cells into the infected calves, and the first detection of marginal bodies in them, was 8 to 10 hours. The generally accepted view that the number of parasitized erythrocytes in acute infection doubles every 24 hours is compatible with the observedy time lapse which occurred before the first marginal bodies appeared in the deer erythrocytes circulating in the bovine vascular system.

Marginal bodies developed in only a small proportion of the newly introduced deer erythrocytes. The deer erythrocytes persisted only a short time in the bovine circulation. The short time available for infection could be responsible for the low infection rate which was seen. It was also reported that deer develop mild and inapparent *Anaplasma* infections.[1, 3] The low infection rate of the deer erythrocytes may therefore be due to a high degree of resistance on the part of the deer erythrocytes.

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SUMMARIO IN INTERLINGUA

Studios in Anaplasmosis. IV. Disveloppamento del Agente Causative in Erythrocytos de Cervo Transfundite in Vitellos

Sanguine ab cervos libere de *Anaplasma* esseva introducite in 2 vitellos con acute infection per *Anaplasma marginale*. Per medio de technicas de tincturation histochimic e immunofluorescente il esseva demonstrate que le organismos de *Anaplasma* penetrava e se disveloppava in le novemente introducite erythrocytos matur de cervo.

The Effect of Vitamin E Administration on Rats Fed Fresh or Autoxidized Beef Tallow

Julius Peter Kreier, V.M.D.; Elwood F. Reber, Ph.D.; Horace W. Norton, Ph.D.

SUMMARY

Rats were fed fresh or autoxidized beef tallow and were given 0.0, 1.75, or 10.0 mg. of vitamin E either orally or intramuscularly.

Vitamin E administration had a significant ($p<0.05$) stimulatory effect on growth. This stimulation occurred both in the rats given fresh tallow and in those given autoxidized tallow in the diet. Those rats fed autoxidized tallow grew significantly less ($p<0.01$) than those given fresh tallow. The growth depression caused by feeding autoxidized tallow was not relieved by the administration of vitamin E. Feeding autoxidized tallow did not affect the red blood cell counts, white blood cell counts, differential counts, or hemoglobin levels of the blood of rats. The autoxidized tallow did not cause testicular damage in the rats to which it was fed. Fat peroxides in the ration were not deposited in the body fat or excreted in the feces.

Autoxidized tallow was digested as efficiently as fresh fat. The route of administration of vitamin E, but not the dose, affected fat digestion. Rats given vitamin E orally excreted significantly more ($p<0.01$) fat than those given vitamin E intramuscularly. Rats given vitamin E intramuscularly did not excrete significantly more fat in their feces than those not given any supplementary vitamin E.

INTRODUCTION

Vitamin E deficiency is associated with a wide variety of pathologic processes. Some of these, such as white muscle disease in calves, encephalomalacia, and exudative diathesis in chicks, stiff lamb disease in sheep, and steatitis in mink, are economically important. In cases of vitamin E-related disorders, the amount, type, and condition of the dietary fat are factors in determining the type of disorder as well as its severity. An understanding of the mechanism of action of vitamin E in combating vitamin E-related disorders is required for a rational approach to prevention and cure of these conditions.

Review of Literature

One theory of the physiologic action of vitamin E ascribes all of its actions to its antioxidant properties and considers the vitamin to be a nonspecific, *in vivo* antioxidant. This theory has been summarized by Dam [3, 4] and Hickman.[5] A second theory of the mode of action of vitamin E, which is discussed by Bowman and Slater,[2] Donaldson and Nason,[6], and Nason, Donaldson, and Lehman,[15] adds a specific role of the vitamin with a specific site of action in one of the respiratory enzyme systems.

Harris and Mason [7] stated that the rate of onset and the occurrence of vitamin E deficiency signs were influenced by oxidative changes accompanying the development of rancidity in cod liver oil and in lard, both of which are commonly used in diets designed to induce vitamin E deficiency. The aggravation of vitamin E deficiency signs is explained on the basis of oxidative destruction of vitamin E in the ration, or after ingestion.

Dam [3] stated that vitamin E deficiency was produced when an animal ingested large quantities of highly unsaturated, easily oxidizable, fatty acids. He suggested that if the fat becomes thoroughly rancid so that the double bonds in the unsaturated fatty acids are destroyed before assimilation, vitamin E deficiency is not produced by feeding the fats. He also indicated that peroxidized fatty acids are not deposited in the body.

In rats, feeding of mildly autoxidized fats causes growth depression.[1] With highly oxidized fats, the reported signs are more severe growth depression, diarrhea, rough hair, prostration, and death.[1, 6]

Luttrell and Mason [14] found that rats fed autoxidized lard and no supplementary vitamin E grew poorly, developed progressive signs of asthenia, anorexia, alopecia, loss of hair luster, and moderate to intense diarrhea. Many animals died. Rats given oral supplementation of vitamin E had no toxic reactions, grew well, and were vigorous.

Received for publication Sept. 26, 1960.

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Taken in part from a thesis submitted by the senior author to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the M.S. degree.

The exact nature of the toxic factors in oxidized oils is still in dispute. A review by the Nutrition Foundation[16] pointed out that some investigators believe that lipid polymers are responsible for the toxic effects.

Materials and Methods

This experiment was designed to study the toxicity of autoxidized beef tallow in the rat, and the effect of administration of vitamin E on that toxicity. The effect of vitamin E administration on growth, feed consumption, and fat digestion of rats fed fresh tallow was also studied. Two experiments were carried out. In the 1st experiment, 20 male weanling rats of the Holtzman strain were fed a ration containing 20% beef tallow (Table 1).

TABLE 1—The Composition of the Ration Used to Determine the Effect of Vitamin E Administration on Rats Fed Fresh or Autoxidized Beef Tallow

Ingredients	Per cent
Casein	25.0
Sucrose	43.5
Salts*	4.0
Brewers' yeast	7.5
Beef tallow, fresh or oxidized	20.0

* Phillips, P. H., and Hart, E. B.: The Effect of Organic Dietary Constituents Upon Chronic Fluorine Toxicosis in the Rat. J. Biol. Chem., 109, (1935): 657–663.

Ten of them were given fresh edible beef tallow. The peroxide number of this tallow was 7.2. The other 10 rats were fed autoxidized beef tallow with a peroxide number of 517. The units for the peroxide number are milliequivalents of peroxide per kilogram of fat. Autoxidation of the tallow was by the method of Andrews *et al.*[1] In gross appearance and odor, the oxidized fat did not differ from fresh fat.

Five of the rats fed fresh tallow and 5 fed autoxidized tallow were given 1.75 mg. of dl-*alpha* tocopheryl acetate by intramuscular injection twice a week. In the other 2 groups, the 5 rats in one were fed fresh tallow and the other 5 were given autoxidized tallow; rats in both groups were each given 1.75 mg. of dl-*alpha* tocopheryl acetate orally twice a week. The orally administered vitamin E was contained in 0.5 ml. of olive oil. The rats were held and licked the vitamin preparation from the tip of a tuberculin syringe. All of the rats were given vitamin A and D by intramuscular injection twice a week. The injected vitamin preparation contained 175 U.S.P. units of vitamin A, 35 U.S.P. units of vitamin D, and sufficient sesame oil to make a total volume of 0.05 ml. The rats which were given vitamin E injections were given 0.5 ml. of olive oil each time vitamins were administered. For injection, the vitamin E was incorporated into the vitamin A and D preparation. The amount of sesame oil was decreased in the vitamin A and D preparations containing tocopherol so that the total volume of material injected was 0.05 ml.

The autoxidized tallow fed to the rats in the 2nd experiment had a peroxide number of 536. The 2nd experiment was a replicate of the 1st experiment, to which 6 supplementary groups were added. The 6 supplementary groups contained 5 rats each. In 2 of the groups, rats of one were fed the fresh tallow and those of the other were fed the autoxidized tallow ration; neither were given supplementary vitamin E. Rats in 2 other groups, one fed each type of tallow, were given 10 mg. of dl-*alpha*-tocopheryl acetate orally twice a week. The rats in the remaining 2 groups, fed each type of tallow, were given 10 mg. of dl-*alpha* tocopheryl acetate by intramuscular injection twice a week.

All the animals were weighed weekly and a record was kept of feed offered and feed not eaten. The rats were fed 3 times a week. Enough feed was offered at each feeding so that there was feed left at the next feeding. Uneaten feed was removed and the bowls were cleaned at each feeding. The rations were stored at −13 C. until fed. At the end of the feeding period, the rats were weighed and killed and their testes were removed and weighed.

Statistical analyses of data from the first experiment were made on the final weight, total feed consumption, and final testes weight At the end of the second experiment, the data from both experiments were combined and analyzed as a unit.

Feces were collected twice during the feeding period of the 2nd replicate; the 1st collection was made during the 6th week and the 2nd during the 8th week.

The feces from the 2 collection periods were analyzed for fat and the total fecal excretion for the period was determined. The fat intake for the period was calculated from the feed consumption records. The data on fat excretion were analyzed statistically by a method which adjusted the results for variations in fat intake.

Sections of the testes from all rats which were fed autoxidized tallow and given 1.75 mg. of vitamin E orally, from those fed autoxidized tallow and given no supplemental E, and from those fed fresh tallow and given 1.75 mg. of vitamin E orally were examined histologically.

The procedure for peroxide determination of fat-containing material was a modification of the method of Lundberg.[13] It involved direct extraction of the fat from the test materials, with the chloroform acetic acid reagent used in the determination.

This modified Lundberg procedure was used to determine the peroxide number of fat from the spermatic cords of all of the rats in the 1st experiment. This method was used to determine the changes in the peroxide number of the ration during storage at −13 C. and to determine the effect on the peroxide number of exposure of the ration in the feed bowls. It was also used to determine the peroxide content of feces.

Results and Discussion

The results of the statistical analyses of the combined experiments confirmed the results of the analyses of the 1st experiment. Therefore, only the results of the

TABLE 2—Data on Rats Fed Fresh or Autoxidized Tallow and Not Given Supplementary Vitamin E or Given Vitamin E Orally or Intramuscularly at 1.75 mg. or 10.0 mg. Twice a Week

Measurement*	Experiment No.	Fresh tallow ration					Autoxidized tallow ration				
		No supplementary vitamin E	Oral supplementary vitamin E		Intramuscular supplementary vitamin E		No supplementary vitamin E	Oral supplementary vitamin E		Intramuscular supplementary vitamin E	
			1.75 mg.	10.0 mg.	1.75 mg.	10.0 mg.		1.75 mg.	10.0 mg.	1.75 mg.	10.0 mg.
Av. body weight (Gm.)	1	.....	281	.....	300	.....	.....	242	.....	267	.....
	2	301	318	314	328	294	216	289	262	254	266
Av. feed consumption (Gm.)	1	.....	693	.....	725	.....	.....	649	.....	711	.....
	2	711	744	699	722	664	596	734	703	683	685
Av. fat consumption (Gm.)	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2	21.8	22.9	22.7	23.9	21.3	18.4	22.0	24.6	22.3	19.9
Av. fat excretion (Gm.)	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2	1.99	2.35	2.19	2.51	1.77	1.69	1.88	2.13	1.82	1.96
Av. % fat in feces	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2	30.8	33.3	40.4	25.3	39.1	37.6	37.3	38.0	30.2	31.8
Av. % fat digested	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2	90.9	89.6	90.4	89.5	91.7	90.8	91.5	91.3	91.8	90.2
Av. weights of pairs of testes (Gm.)	1	.....	3.00	.....	2.95	.....	.....	2.74	.....	2.96	.....
	2	3.15	3.17	3.15	3.17	3.15	2.75	3.12	3.04	2.84	2.74

Body weight is weight at the end of the 8-week experimental period. Feed consumption is total consumption for the 8-week experimental period. Data on fat consumption and fat excretion and per cent of fat in feces were recorded during the 2 1-week collection periods. The data on per cent of fat digested were calculated from the fat consumption and excretion figures.

* Five animals in each group.

analyses of the complete experiment are reported.

Growth depression of the rats fed the ration containing autoxidized tallow was found to be highly significant ($p<0.01$) when compared with that of rats given the fresh tallow ration. This significance was not decreased by adjustment for feed consumption. The growth depression of the rats fed the autoxidized tallow was not significantly less in those given vitamin E by either route or at either level of administration.

The administration of vitamin E affected the final weight of the rats. Those rats given supplemental vitamin E were significantly heavier ($p<0.05$) than those which were not given any. However, those given the larger amount of vitamin E were not significantly heavier than those given the smaller amount (Table 2).

The testes from the rats fed autoxidized tallow in their diet and no supplementary vitamin E did not differ histologically from those of rats given vitamin E and fresh tallow orally. In all cases, normal spermatozoa were present and the germinal epithelium was normal.

The testes of the rats fed autoxidized tallow were significantly ($p<0.01$) smaller than those of rats fed fresh tallow. This reduction, however, was correlated ($p<0.01$) with general body size reduction. Feed consumption was not significantly affected by either the type of fat fed or the amount or route of vitamin E given.

The red blood cell counts, white blood cell counts, differential counts, and hemoglobin contents of the blood were determined on all of the animals in the 1st experiment, once before assignment and again during the 7th experimental week. Neither the composition of the ration nor the route of administration of vitamin E affected the results of these determinations.

Fat tissue from the spermatic cords of all rats in the 1st experiment was examined for fat peroxides. The fat tissue from 2 rats fed autoxidized tallow and given vitamin E by injection and from one fed autoxidized tallow and given vitamin E orally contained fat peroxides. The amounts were, respectively, 0.8, 1.1, and 1.1 mEq. of peroxide per kilogram of fat tissue.

Peroxides were not detected in fresh feces from 2 rats fed the autoxidized ration or from 2 fed the fresh tallow ration.

The percentage of fat in the feces of the rats given vitamin E orally was significantly greater ($p<0.05$) than that in the feces of the rats given vitamin E by injection. When the percentage of fat in the feces of rats given vitamin E orally or intramuscularly was compared with the percentage of fat in the feces of those not given supplementary vitamin E, no significant differences were found. The mean value of the percentage of fat in the feces of the rats given no supplementary vitamin E was about halfway between the mean values of the other 2 groups.

The results of the statistical analysis of the fecal fat excretion data indicated that the autoxidized tallow was digested as well as the fresh tallow, and that the amount of vitamin E given did not affect fat excretion. The route of administration of the vitamin E significantly ($p<0.01$) affected the fat excretion. More fat was excreted by rats given vitamin E orally than by those given the vitamin intramuscularly. There was also more fat in the feces of animals given vitamin E orally ($p<0.01$) than in the feces of those given no supplementary vitamin E.

The results of this experiment indicate that autoxidation causes changes in beef tallow, making it toxic to rats. The sign of this toxicosis was growth depression. This result is in agreement with results of other experiments which have shown that various autoxidized oils are toxic.[1, 9-12] Andrews *et al.*[1] tested autoxidized soybean oil for toxicity. Soybean oil with a peroxide number comparable to that of the tallow fed in this experiment caused growth depression.

This experiment showed no interaction between the effects of administering vitamin E and of feeding autoxidized tallow. Luttrell and Mason [14] showed that rats fed vitamin E did not develop signs of toxicosis when fed autoxidized lard, while rats given autoxidized lard and no vitamin E grew poorly, and some died. It is difficult to compare the 2 experiments, because Luttrell and Mason [14] did not chemically define the degree of autoxidation of the tallow fed, nor did they accurately define the potency of the vitamin E preparation given.

Signs of vitamin E deficiency did not develop in the rats fed the autoxidized tallow, not even in those which were not given supplementary vitamin E. This result is in agreement with the statement by Dam [3] that vitamin E deficiency is not caused by feeding oxidized fats. The lack of effect of vitamin E in reversing the toxic effect of

the autoxidized tallow indicates the importance of products of oxidation, other than lipid peroxides, as causes of toxicity of autoxidized fat. The feeding of autoxidized tallow obviously did not interfere with appetite, since there was no significant difference in feed consumption among the groups of rats.

Analysis of the growth data showed that all of the rats given supplementary vitamin E grew better than those not given any supplement. The higher level of administration of the vitamin did not stimulate growth more than did the lower level. The route of administration of the vitamin did not alter the effect. These results suggest that vitamin E may have a growth-stimulating effect.

Orally administered vitamin E has been reported to be effective in preventing vitamin E deficiency while intramuscularly administered vitamin E was not.[5] The effect of vitamin E on fat excretion occurred only when the vitamin was given orally. These results suggest a local site of action for vitamin E in the digestive tract.

It may also be concluded that the body destroys most fat peroxides which are eaten, since (1) only a small amount of peroxide was present in the tissues of rats given the autoxidized fat, and (2) the feces of the rats did not contain peroxides.

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SUMMARIO IN INTERLINGUA

Le Effecto del Administration de Vitamina E in Rattos Alimentate con Stear Bovin Fresc o Autoxydate

Rattos esseva alimentate con stear bovin fresc o autoxydate e esseva tractate con 0, 1,75, o 10 mg de vitamina E per via oral o intramuscular.

Le administration de vitamina E habeva un significative effecto stimulatori in le crescentia ($p<0,05$). Iste stimulation occurreva tanto in le rattos recipiente stear fresc in lor dieta como etiam in illos recipiente stear autoxydate. Le rattos alimentate con stear autoxydate habeva, in comparation con le rattos alimentate con stear fresc, un crescentia significativemente inferior ($p < 0,01$). Le depression del crescentia causate per le alimentation con stear autoxydate non esseva alleviate per le administration de vitamina E. Le alimentation con stear autoxydate non afficeva le numeration erythrocytic, le numeration leucocytic, le numeration differential, o le nivello de hemoglobina in le sanguine del rattos. Le stear autoxydate non causava damno testicular in le rattos que esseva alimentate con illo. Peroxydos grassiose in le dieta non esseva deponite in le grassia corporee e non excernite in le feces.

Stear autoxydate esseva digerite tanto efficacemente como grassia fresc. Le via de administration de vitamina E, sed non le dose de illo, afficeva le digestion de grassia. Rattos recipiente vitamina E per via oral habeva, in comparation con illos recipiente vitamina E per via intramuscular, un excretion de grassia significativemente superior ($p<0,01$). Rattos recipiente vitamina E per via intramuscular non excerneva significativemente plus grassia in lor feces que rattos non recipiente ulle vitamina E supplementari.

Leptospirosis in Hamsters on Diets Containing Various Levels of Riboflavin

R. E. Smith, V.M.D., and I. M. Reynolds, M.S.

SUMMARY

Riboflavin deficiency in hamsters was characterized primarily by reduced appetite and loss of weight. Leptospirosis *(L. pomona)* in hamsters varied from an acute highly fatal infection with hemorrhage and icterus to a subclinical disease manifested by chronic nephritis. The dietary level of riboflavin (0 to 100 mg/kg. of feed) failed to influence the course of leptospirosis in hamsters.

INTRODUCTION

Much interest has developed in recent years in the influence of nutrition on infection, and the ability of the level of nutrition to enhance resistance or increase susceptibility depending upon the specific disease involved. The role of vitamins in infection has been explored by many investigators. Hill and Garren [5] demonstrated resistance in chicks to fowl typhoid when fed known required vitamins at high level. Seronde [8] showed that rats deficient in pantothenic acid developed a spontaneous *Corynebacterium* infection. Axelrod and Pruzansky,[1] working with albino rats, showed that antibody response can be inhibited by deficiencies of various vitamins, many of them members of the vitamin B complex.

The present study was suggested by 2 observations concerning equine periodic ophthalmia (recurrent iridocyclitis). Jones *et al.*[6] demonstrated that horses, whose rations were supplemented with riboflavin, had a lower incidence of periodic ophthalmia than did controls with nonsupplemented rations. Bryans [2] reported the findings of many investigators that the majority of horses with clinical signs of periodic ophthalmia react positively to serologic tests for leptospirosis. The question arose as to the possible relationship between the dietary level of riboflavin and the development of leptospirosis.

Hamsters were selected as experimental animals due to their susceptibility to leptospirosis. Hamdy and Ferguson [3] reported the effects of serial passage of a strain of *Leptospira pomona* in hamsters. Hamilton and Hogan [4] reported that hamsters require dietary riboflavin. Mann *et al.*[7] stated that manifestations of riboflavin deficiency are notably nonspecific and, in most species, are concerned with weight loss and dermatitis. No reports were found which described the effects of riboflavin deficiency upon hamsters. Accordingly, studies were planned to: (1) determine the effects of riboflavin deficiency and supplementation upon hamsters; (2) further study leptospirosis in hamsters; and (3) find if a relationship exists between various levels of dietary riboflavin and the pathogenesis of leptospirosis in hamsters.

Materials and Methods

Hamsters in these trials were purchased from 2 sources, those used in early trials from Golden Nugget Hamstery, Maynard, Mass., and in later trials from Bio-Research Consultants, Cambridge, Mass. Hamsters used were 4 to 6 weeks old (50 to 60 Gm.) and were of the same sex and age for each trial.

Riboflavin-deficient diet* was composed as follows:

BASIC MIXTURE

Vitamin test casein	18%
Sucrose	68%
Vegetable oil	10%
U.S.P. salt mixture No. 2	4%

Received for publication July 18, 1960.

From the Department of Veterinary Science, University of Massachusetts, Amherst. Published as contribution No. 1253, Massachusetts Agricultural Experiment Station.

This investigation was supported in part by Hatch funds under project No. 141.

The authors thank Mr. G. W. Clark for technical assistance, and Dr. M. Sevoian for assistance in preparation and interpretation of histopathologic specimens.

* Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

VITAMINS ADDED PER 100 LB. OF BASIC MIXTURE

Vitamin A concentrate (200,000 units per gram)	4.5 Gm.
Vitamin D concentrate (400,000 units per gram)	0.25 Gm.
Alpha tocopherol	5.0 Gm.
Ascorbic acid	45.0 Gm.
Inositol	5.0 Gm.
Choline chloride	75.0 Gm.
Menadione	2.25 Gm.
P-aminobenzoic acid	5.0 Gm.
Niacin	4.5 Gm.
Pyridoxine hydrochloride	1.0 Gm.
Thiamine hydrochloride	1.0 Gm.
Calcium pantothenate	3.0 Gm.
Riboflavin	None
Biotin	20.0 mg.
Folic acid	90.0 mg.
Vitamin B_{12}	1.35 mg.

In order to shift hamsters from a pelleted laboratory ration to the granulated purified diet used in these trials, a 2-week adjustment period was required. During this period, the pelleted ration was fed in ground form, and then mixed in gradually decreasing quantities with the purified diet until the purified diet alone was fed. Hamsters purchased at 4 weeks of age were thus 6 weeks old when each trial began.

Riboflavin* was used for oral supplementation. Riboflavin-5′-phosphate sodium* was used for subcutaneous administration. The dose of 50 μg. of riboflavin for daily subcutaneous inoculation was determined by the process of elimination. When 100 μg. of riboflavin or higher dosage was given, occasional deaths occurred after one week. In these cases, the kidneys were primarily involved and appeared greatly swollen and bright yellow.

Hamsters were housed individually in a battery of wire-bottomed cages. All hamsters were weighed at weekly intervals, and weights were recorded to the nearest half gram.

Leptospiral cultures of *L. pomona* for inoculation were identified as follows: *L. pomona*, EHB, originally isolated from swine fetuses by Dr. E. H. Bohl of Ohio State University, was hamster-adapted and supplied by Dr. A. H. Hamdy of the Ohio Agricultural Experiment Station; *L. pomona*, MLS, originally isolated from cattle, had been maintained in guinea pigs from the time of its isolation. This culture was supplied by Dr. J. A. Baker of Cornell University and maintained in hamsters after receipt. Two cultures were necessary due to loss of virulence in the 1st (EHB strain).

Hamdy and Ferguson[8] reported on hamster adaptation of *L. pomona*, Hardacre strain, by serial passage of infected blood, and on the pathologic changes found in hamsters. Both strains of *L. pomona* used in the present studies had essentially the same characteristics, except for differences arising from changes in virulence.

Leptospira pomona, EHB, had been serially passed 35 times in hamsters when received and produced death in 6 days. After 35 more passages, virulence gradually declined until deaths were not produced until 16 days, and finally no deaths occurred. Passages were made by inoculation intraperitoneally of 0.1 to 0.2 ml. of infected blood which had been collected by cardiac puncture on the 3rd or 4th day postinoculation (PI). Infected blood was inoculated into Schuffner's medium at the time of collection to prove successful transfer of leptospires. The presence of infection in late passages was detected by positive blood cultures and positive serologic reaction 2 to 3 weeks PI.

Leptospira pomona, MLS, had been maintained in guinea pigs from the time of its original isolation. During the course of these studies, it was maintained through 130 hamster passages. It produced death on PI day 4 or 5 and showed no decline in virulence.

While infected hamster blood was used as inoculum in serial passages, a 7-day culture of infected hamster blood in Schuffner's medium was used as inoculum in experimental trials. Culture for 7 days in artificial mediums did not appear to seriously reduce virulence. Further passage in artificial mediums, however, reduced virulence markedly.

Silver impregnation techniques were used to demonstrate leptospires in tissue. Both Warthin-Starry and Levaditi staining techniques were used. In this study, the former technique showed leptospires more frequently and with less distortion. This method also allowed direct comparison with similar sections stained by the hematoxylin-eosin method. All silver-stained preparations were examined with widefield eye pieces at 1,455 magnifications.

Results

RIBOFLAVIN DEFICIENCY IN HAMSTERS

Eighteen female hamsters 6 weeks old were used in this trial. Twelve were given a riboflavin-deficient diet, and 6 were given the same diet with riboflavin added at the level of 20 mg./kg. of feed. This was the level at which riboflavin would be included in the complete purified diet. After 8 weeks, the deficient group was divided. Half were given the 20 mg. ration, and the other half continued on the deficient ration. All were killed at 12 weeks. The effect of riboflavin on weight gain is shown graphically (Fig. 1).

The deficient hamsters were stunted, dull, and inactive. Their coats lacked luster, but no skin lesions developed during the test period. No eye disturbances were observed. Reduction in food and water intake was constant, and 3 deaths occurred in the deficient group at 8 weeks, probably from inanition.

At necropsy, deficient hamsters were emaciated and stunted, with all organs pro-

* Purchased from Hoffmann-LaRoche, Inc., Nutley, N.J.

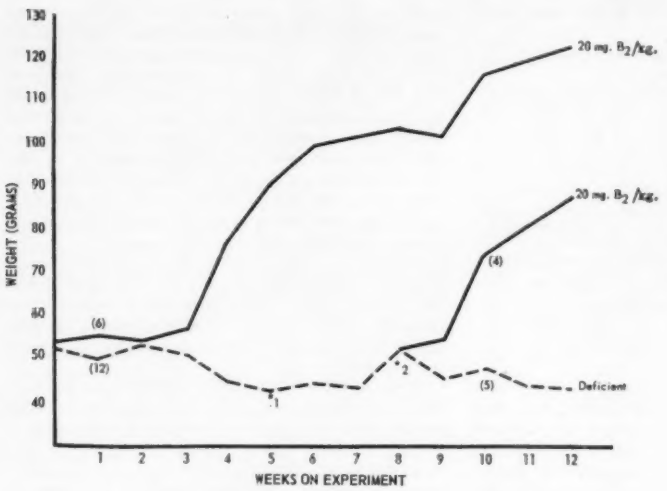

Fig. 1—Growth chart of riboflavin-deficient and -supplemented hamsters. Asterisk indicates deaths; numbers in parentheses indicate the number of hamsters.

portionately reduced in size, but no gross lesions were apparent. Histologic examination of lungs, livers, spleens, kidneys, and eyes was negative for microscopic changes.

Supplemented hamsters were active and alert and made consistent gains in weight after a 2-week lag. This length of time was necessary for hamsters to become accustomed to the purified diet. The coat was glossy, short, and dense. At necropsy, fat accumulations in perirenal and pelvic areas testified to their good condition. No gross or microscopic changes could be demonstrated.

Those deficient hamsters which were returned to the 20 mg. diet after 8 weeks gained weight immediately and improved in general condition. At necropsy, they were intermediate to the deficient and supplemented groups in appearance.

Stunting and failure to gain weight were used as criteria of riboflavin deficiency in later trials. Six to 8 weeks were allowed for the deficient state to become established.

LEPTOSPIROSIS IN HAMSTERS

Hamster passage of *L. pomona* and its inoculation in repeated trials with normal hamsters afforded ample opportunity to study the effects of leptospirosis.

As in many other species, the disease varied from an acute, highly fatal infection with hemorrhage and icterus to a subclinical disease manifested only by chronic nephritis.

The course is believed to vary primarily with the virulence of the organism. Efforts to count motile leptospires yielded approximations only. Where 7-day culture had been used as inoculum in 0.1-ml. to 0.5-ml. amounts, even the smallest quantities represented millions of leptospires. Where virulence had been reduced by culture, the death pattern became extended and allowed resistance of the host to influence the course. Variations in virulence appeared in 7-day blood cultures from different hamster passages. This factor necessitated many trials in these studies since the degree of virulence of the inoculum could not be anticipated.

Clinically, *L. pomona* infection in hamsters was characterized by few signs during the first 3 or 4 days PI. The hamsters were active and appetite was undisturbed. Approximately 24 hours before death, signs of icterus could be detected on the foot pads. Urine appeared yellow to orange. Hemoglobinuria was not observed. Hyperesthesia and finally clonic convulsions were terminal signs. Death usually occurred on PI day 4 or 5. If the organism failed to produce death during the 1st week, clinical signs were usually absent. Death was postponed for several weeks, and then was due to the effects of chronic nephritis.

The lesions present varied with the course of the disease. In hamsters dead on PI day 4 or 5, gross lesions consisted of hemorrhages and icterus. Petechial hemorrhages or gross hemorrhages in the lung seemed

characteristic. Icterus varied from mild to severe, or from a light yellow to orange. The liver, spleen, and kidneys were dark red and swollen, and the kidneys frequently contained scattered petechial hemorrhages.

As the course was prolonged to 7 or 8 days, lesions were less severe. Petechial hemorrhages were present in the lungs. The liver appeared grossly normal. The spleen was pale pink. The kidneys were enlarged but pale, even cream-colored in some instances, and petechiae were frequently absent. Icterus was mild to moderate.

In those that died on PI days 10 to 12, the lungs still had petechiae, but fewer and smaller other hemorrhages were present. The liver was grossly normal. The spleen was pale pink. The kidneys were pale but normal in size, with occasional pits seen on the cortical surface. Icterus was mild or absent.

Those hamsters which survived and were killed at 3 to 4 weeks PI had gross lesions confined to the kidneys. Kidneys were pale and normal in size or shrunken. The cortical surface was extensively pitted, mulberry-like in severe cases. The lungs, liver, and spleen were grossly normal, and icterus was absent.

The results of histopathologic examination supported the gross findings. In acute leptospirosis, the lungs contained scattered areas of hemorrhage and atelectasis. The liver was congested, and the kidneys were congested and hemorrhagic.

In hamsters with chronic cases, changes were confined to the kidneys. These included distention of Bowman's capsule, dilation of proximal tubules, degeneration of tubular epithelium, amorphous or cellular casts, and scattered areas of lymphocytic infiltration and fibroblast proliferation. In addition, there were small areas of necrosis with calcification in most severe cases. Hamsters which had renal necrosis and calcification usually died 2 to 4 weeks PI.

Although nervous signs were part of the clinical syndrome, the central nervous system was not subjected to histologic examination.

In hamsters with acute cases, leptospires were seen scattered among the cords of liver cells, outside the proximal tubules and in capillaries of the kidney, and occasionally either singly or in clumps in pulmonary vessels. The liver, however, contained the greatest concentration of organisms.

In hamsters with chronic cases, leptospires could only be found in the kidneys. At this time (3 weeks PI) they appeared as hairlike masses lining the proximal tubules. In kidneys which had areas of calcification, no leptospires were found.

In the trials which follow, a comparison is made of the effects of various levels of riboflavin on the development of leptospirosis. Typical lesions and positive serologic reactions served as criteria of infection.

LEPTOSPIROSIS IN RIBOFLAVIN-DEFICIENT AND -SUPPLEMENTED HAMSTERS

In a pilot trial, deficient hamsters inoculated with leptospires died earlier than supplemented hamsters. Food intake of the deficient hamsters was poor, and starvation was believed to influence the course. Therefore, starvation controls were included in the experimental design.

In each of 8 trials, 72 hamsters were distributed equally among 6 groups. Hamsters in 2 groups (A and B, Table 1) were fed a purified diet deficient in riboflavin; at the time of inoculation, those in group B were given, in addition, 50 μg. of vitamin B_2 daily, administered subcutaneously. Purified diet fed to animals in groups C and D was supplemented with 20 mg. and 100 mg. of vitamin B_2/kg. of feed, respec-

TABLE 1—The Course of Leptospirosis in Hamsters on Various Dietary Levels of Riboflavin (0 to 100 mg./kg. of Feed) in Trial C

Dietary groups	Total hamsters	Death pattern (days PI)														Killed (days PI)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	21
A—Riboflavin deficient	8	..	..	..	..	3	..	1	1	..	..	2	..	..	..	1*
B—Riboflavin deficient + 50 μg. B_2	8	..	..	..	..	..	3	1	..	..	..	2	1	..	..	1*
C—20 mg. B_2	8	..	..	..	..	..	..	1	3	..	..	2	..	1	..	1*
D—100 mg. B_2	8	..	..	..	..	1	1	1	2	..	1	1	1	..	..	..
E—Starvation	8	..	..	..	..	2	2	..	..	..	2	1	..	1	..	..
F—Starvation + 50 μg. B_2	8	..	..	..	..	2	3	2	..	1	..	..	..	..	..	..
Controls (4 for each group above)	24	..	..	..	..	..	..	..	..	..	..	..	..	..	..	24**

* Positive for lesions and antibodies; ** negative for lesions and antibodies, PI = postinoculation.

tively. These 4 groups were fed *ad libitum*. Those in groups E and F, which served as starvation controls, were fed a limited amount of the basal diet containing 20 mg. of vitamin B_2/kg. of feed; at the time of inoculation, hamsters in group F were given an additional 50 μg. of vitamin B_2 daily, administered subcutaneously.

Each of the 6 groups of 12 hamsters included 8 hamsters intended for leptospiral inoculation and 4 for uninoculated controls. After a 6- to 8-week depletion period, which allowed signs of riboflavin deficiency to develop freely, cultures of leptospires were inoculated into 8 hamsters of each group.

The trials which follow are representative of the results obtained with leptospiral inoculums of varying virulence.

Trial A.—Seventy-two 6-week-old female hamsters were started in this trial and, after a 6-week depletion period, 8 hamsters in each group were inoculated intraperitoneally with 0.5 ml. of a 7-day hamster blood culture of *L. pomona*, EHB strain.

No deaths occurred during the first 3 weeks PI, and all hamsters were exsanguinated at the end of this period. The serologic titers, determined by Stoenner's capillary tube test,[9] fell in the range of 1:160 to 1:640 and no significant effect of riboflavin on antibody response could be demonstrated. No gross lesions were seen at necropsy. Inoculated hamsters were apparently only vaccinated by the culture used.

Trial B.—Seventy-two 6-week-old female hamsters were started as in the above trial. After a 6-week depletion period, the hamsters to be inoculated were given 0.5 ml. of a 7-day hamster blood culture of *L. pomona*, MLS strain.

With the exception of one infected hamster (in the group given 20 mg. of vitamin B_2) which lived 9 days, all other inoculated hamsters died on PI day 5 or 6. All had lesions of acute leptospirosis at necropsy. The high virulence of the culture precluded any attempts to determine the influence of riboflavin on the death pattern.

Trial C.—Seventy-two 6-week-old male hamsters were used. Those to be infected were inoculated intraperitoneally after a 6-week depletion period with 0.1 ml. of a 7-day hamster blood culture of *L. pomona*, MLS strain.

Of the 48 infected hamsters, 45 died between PI days 5 and 13. Survivors were killed PI day 21. The 2 deficient groups and the 20 mg. group each had one survivor at this time. These 3 hamsters were serologically positive and were found to have chronic nephritis at necropsy. The death pattern in this trial is shown (Table 1) and indicates the results produced by an inoculum of moderate virulence.

Uninoculated Controls.—In all trials, uninoculated hamsters were killed at the same time as infected survivors (21 days PI) or 9 to 11 weeks from the beginning of each experiment.

The riboflavin-deficient controls appeared stunted and failed to gain, but no deaths occurred. The riboflavin-deficient controls given vitamin B_2 subcutaneously each day after the 6-week depletion period had a gradual and continuous increase in appetite and weight gain.

The starvation controls, maintained by limited feeding of the 20-mg. diet at the approximate weights of the deficient controls, were more active and alert than the deficient animals. The starvation controls given vitamin B_2 subcutaneously developed no gain in weight over the nonsupplemented starvation group.

The 20-mg. controls were in good condition and continually gained weight. The 100-mg. controls usually outweighed the other groups and were often obese at necropsy.

All controls were serologically negative. No lesions were apparent at necropsy. Weights of controls in trial C are representative of all trials and are shown (Fig. 2).

Discussion

While littermate controls were not used in these experiments, the possible error is at least partially overcome by finding comparable results in repeated trials with hamsters from 2 unrelated sources.

Three deaths within 8 weeks in the initial trial with riboflavin deficiency are believed due to inanition brought about by refusal to eat the diet. This is further substantiated by the fact that no deficient controls were lost in other trials during a 9- to 11-week depletion period.

Starvation controls were necessary since the absence of riboflavin seriously reduced the appetite and food intake. Deficient controls given vitamin B_2 subcutaneously in 50-μg. daily amounts after a 6-week depletion period had an immediate improvement in appetite, food intake, and weight gain. Starvation controls, whose appetite

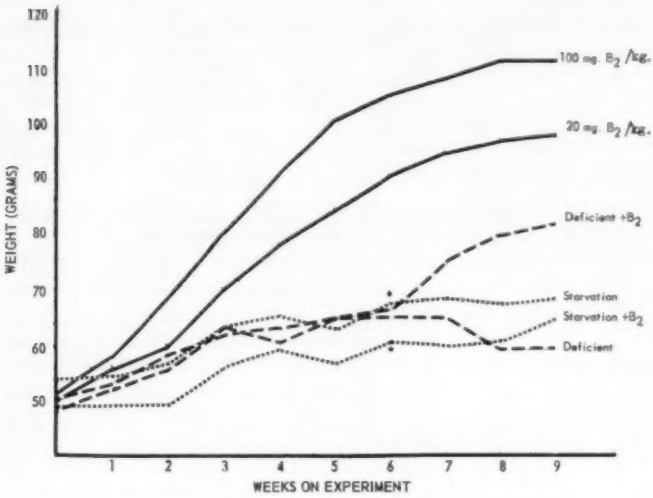

Fig. 2—Growth chart of uninoculated controls in trial C. Asterisk indicates that 5 μg. of vitamin B_2 was given subcutaneously each day. There were 4 hamsters in each group.

was ravenous from the limited food available, had no increase in weight gain following the daily subcutaneous inoculation of 50 μg. of riboflavin.

In line with the view of Mann *et al.*[7] that signs of vitamin B_2 deficiency in many species are nonspecific, riboflavin deficiency in hamsters was characterized primarily by decrease in appetite and failure to gain weight. Had these trials extended over a longer period, deaths would undoubtedly have occurred from inanition, riboflavin deficiency, or an over-all vitamin depletion from reduced food intake.

The selection of hamster tissues for histologic examination was based on those organs which might be expected to have lesions of leptospirosis; *i.e.*, lungs, liver, spleen, kidneys, and eyes. The presence or absence of riboflavin appeared to have no effect on the tissues examined.

A fluctuation in virulence in serial passage of an *L. pomona* strain in hamsters was reported by Hamdy and Ferguson.[3] Virulence increased in early passages and then began to decrease as judged by hamster deaths. A similar pattern was shown for the *L. pomona*, EHB strain used in early trials in this study. Received initially from Dr. Hamdy, this strain was a different isolate from that used in his experiments. Conversely, the MLS strain of *L. pomona*, used in later trials in this series, has had no loss of virulence in 2 years of hamster passage, with deaths generally occurring 4 to 5 days PI. Infected hamster blood from recent passages of this strain has killed sheep with typical hemolytic anemia and icterus.

Lesions of acute and chronic leptospirosis found in this study coincided with those described by Hamdy and Ferguson.[3] It was possible to trace a more gradual change in gross lesions in this study with deaths spread over a 3-week period. While hemoglobinuria is frequently observed in acute leptospirosis in ruminants, and was also reported by Hamdy and Ferguson [3] in hamsters, this sign was not observed in these experiments.

Axelrod and Pruzansky [1] showed that antibody response in albino rats could be markedly inhibited in various vitamin-deficient states. The antigen used was diphtheria toxoid, and inhibition of antibody response occurred in rats deficient in pantothenic acid, pyridoxine, riboflavin, biotin, and vitamin D. In this study with riboflavin-deficient and -supplemented hamsters, no significant difference in antibody level could be demonstrated. In the routine passage of leptospires in hamsters to maintain virulence, these hamsters which have developed chronic leptospirosis have had the same antibody level (1:160 to 1:640) when killed 3 weeks PI.

The inoculum for each trial necessarily represented a different hamster passage of *L. pomona*. The control of virulence under these circumstances presented difficulty. In

separate trials, dilution of cultures or chicken embryo passage did not uniformly lengthen the course in hamsters. Cultivation in artificial mediums, Schuffner's modification of Verwoort's medium, was effective in reducing virulence. In all cases, virulence could not be predicted prior to the actual use of the inoculum in a given trial.

The death pattern in hamsters was used as the measurement of the effect of riboflavin levels on the pathogenesis of leptospirosis. In these trials, no significant influence of riboflavin on the death pattern could be demonstrated.

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SUMMARIO IN INTERLINGUA

Leptospirosis in Hamsters Mantenite con Dietas Continente Varie Nivellos de Riboflavina

Carentia de riboflavina in hamsters esseva characterisate primarimente per un reducite appetito e perdita de peso. Leptospirosis *(Leptospira pomona)* in hamsters variava ab un acute e altemente mortal infection con hemorrhagia e ictero ad un morbo subclinic manifeste per chronic nephritis. Le nivello dietari de riboflavina (0 a 100 mg per kg de nutritura) non influentiava le curso de leptospirosis in hamsters.

A Rapid, Permanent Wright's Staining Method for Chromosomes and Cell Nuclei

Haskell S. Tubiash, M.S.P.H.

SUMMARY

In devising a procedure for rapidly staining chromosomes and nuclei of cells cultured on glass and in suspensions, preparations fixed by acetic alcohol and air-dried were neutralized with ammonia water or sodium bicarbonate, treated with serum, and stained by Wright's method. Cells cultured from ovine, bovine, and murine tissues were successfully stained. These preparations are permanent; they may be examined by conventional microscopy and photographed at the convenience of the investigator.

INTRODUCTION

The establishment and propagation of increasing numbers of cell lines and primary cell cultures for research in virology, cytology, oncology, radiation biology, and other disciplines, in addition to genetics, has broadened interest in the chromosomal components of cells. It is believed that changes in ploidy reflect corresponding changes or transformations in many of the characteristics of these cells.

Conventional methods for visualizing chromosomes usually employ stained, wet preparations.[3] These are made after treatment to spread the chromosomes through exposure to hypotonic solutions, followed by squashing or air-drying. The life of these temporary preparations may be extended by sealing the coverslip with petroleum jelly or Krönig's cement. These wet preparations are best observed by phase microscopy and can be rendered permanent by the dry ice freezing method of Conger and Fairchild[1] or by glycerine infusion as described by Rattenbury.[4] Properly prepared squash specimens may also be stained by Feulgen procedures.[2]

A method using Wright's stain has been developed in this laboratory for rapid permanent staining of chromosomes and nuclei of cells in tissue culture.

Materials and Methods

Chromosomes.—Actively proliferating cell cultures are prepared on slides or cover slips and treated in the conventional manner with colchicine and hypotonic NaCl solution.[6] The glass-adherent cells are then fixed in acetic alcohol and air-dried according to the method of Rothfels and Siminovitch[5] or Tjio and Puck.[6]* Cells from suspended cultures may also be fixed, spread on glass, and air-dried.[5] Air-dried slides may be retained indefinitely prior to further processing.

Instead of proceeding with conventional temporary wet acetic-orcein or acetic-carmine stains, air-dried preparations can be stained permanently in the following manner. Slides are neutralized in ammonia water (2 to 3 drops concentrated ammonium hydroxide to a Coplin jar containing 50 ml. of distilled water) or 2% aqueous sodium bicarbonate solution for 1 minute. They are drained briefly, dipped wet into serum, drained, and air-dried in a vertical position. Staining is by Wright's method in a manner similar to the processing of blood smears. The procedure is completed by thorough washing in water and air-drying.

Preparations illustrated (Fig. 1–6) were neutralized in 2% sodium bicarbonate, treated with bovine serum, and exposed to undiluted Wright's stain for 1 minute. The stain was diluted with approximately 2 volumes of diluent, and staining was continued for an additional 3 minutes. Phosphate-buffered water was used as the diluent.[7]

Nuclei.—Excellent stains of cells proliferating on slides or cover slips may be produced by eliminating exposure to hypotonic saline solution and reducing the period of fixation in acetic alcohol. Glass-adherent cells on slides or cover slips are removed from growth medium, washed in phosphate-buffered saline solution, fixed for 2 to 5 minutes in acetic alcohol (according to the density

Received for publication Oct. 21, 1960.

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The technical assistance of Mr. Albert Paolantonio in the processing of photomicrographs is acknowledged with thanks. Ovine and murine cell lines were supplied through the courtesy of Dr. William R. Hess.

* Preparations illustrated were grown on slides under 5% CO_2 tension in Petri dishes for 24 to 48 hours. Chromosome preparations were incubated in colchicine (1:400,000) for 1 to 4 hours and in ½ normal hypotonic NaCl solution for 45 minutes to 1 hour. They were then fixed in acetic alcohol (1:3) for 10 minutes and air-dried in a horizontal position.

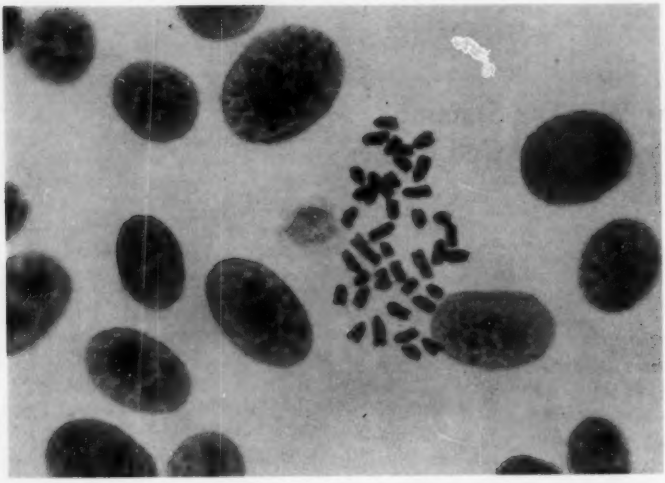

Fig. 1—Transmissible cells of mouse tumor origin. Diploid Chromosomes of a cell in metaphase surrounded by nuclei of interphase cells. Modified Wright's stain; x 600.

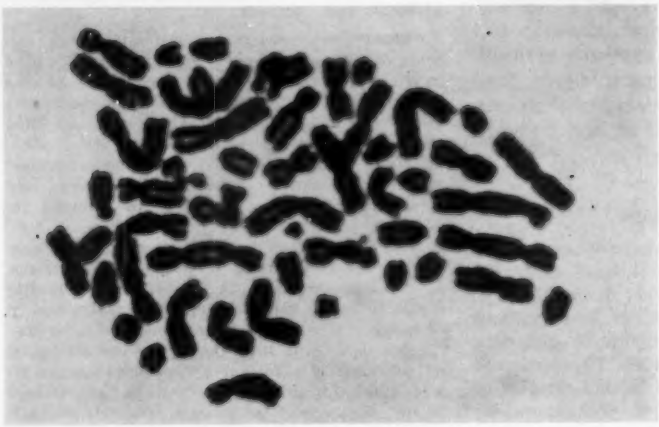

Fig. 2—Transmissible cell of lamb testis origin. Aneuploid chromosomes in metaphase. Modified Wright's stain; x 1000.

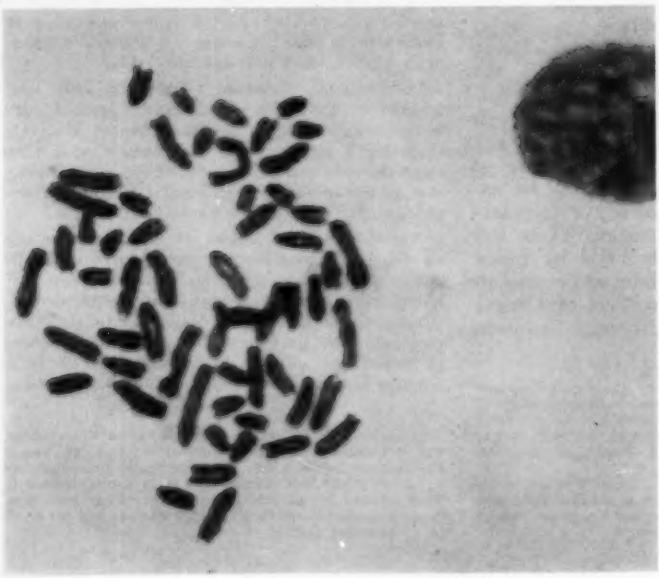

Fig. 3—Diploid chromosomes of bovine kidney cell in metaphase. Primary culture. Modified Wright's x 800.

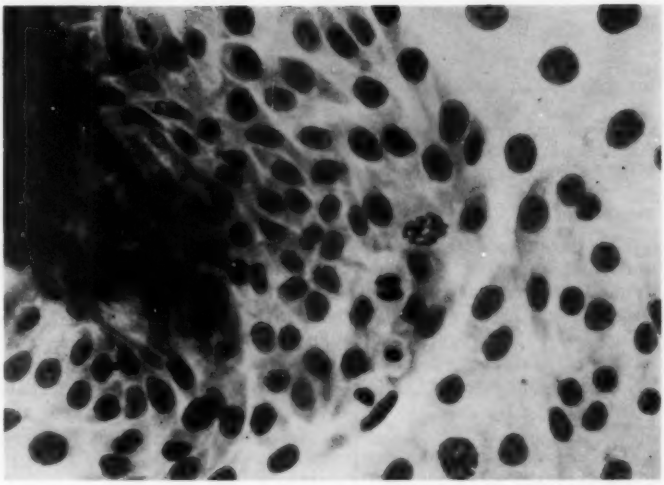

Fig. 4—Bovine kidney cells. Primary culture. Modified Wright's stain; x 150.

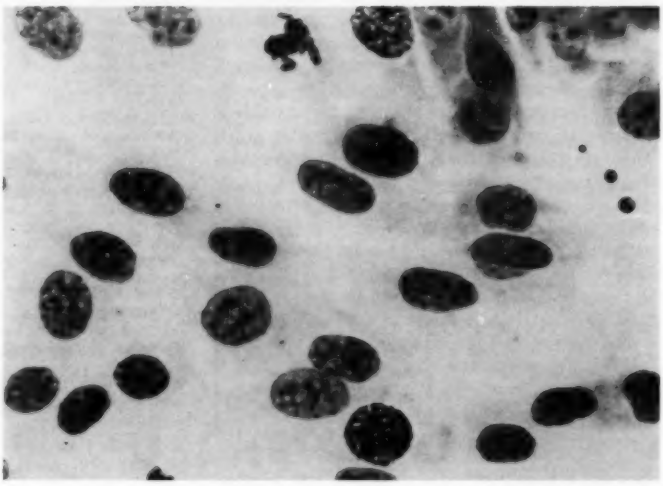

Fig. 5—Bovine kidney cells. Primary culture. Modified Wright's stain; x 270.

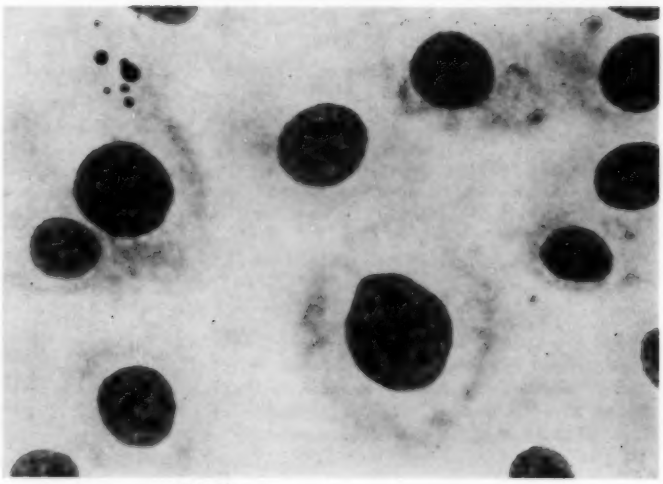

Fig. 6—Bovine kidney cells. Primary culture. Modified Wright's stain; x 600.

of the cellular growth), and air-dried. They are then treated with sodium bicarbonate, serum, and Wright's stain as previously described.

Results

Chromosomes and cell nuclei of primary and line cultures derived from bovine, ovine, and murine tissues have been successfully stained by the methods outlined (Fig 1–6). The preparations are permanent and can be examined by ordinary brightfield microscopy.

Discussion

The staining method described can be utilized with materials and optical equipment readily available in any biological laboratory. Bovine serum was used in the preparations illustrated, but any serum conveniently available may be utilized. While serum sterility is not necessary, gross contamination should be avoided. Bacterial contaminants take up the stain and may be confused with chromosomes. The film of serum on the cells minimizes distortion of cellular elements and prevents stain precipitate which tends to form when serum is not used. It also intensifies the blue-violet basophilia of the chromatin and improves visualization of the chromosomes.

Where preparations are designed for counting and characterizing chromosomes, factors limiting the quality of the final product appear to be governed by the density of the glass-adherent cells and the success of the fixing-spreading procedure rather than by the properties of the described staining method. After acetic alcohol fixation and air-drying, slides can be retained for any desired period of time before further processing. The same condition holds true after serum treatment. Unlike temporary wet preparations, these slides can be accumulated, stained, and photographed at convenience. They can, of course, be retained as permanent collections for future reference.

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SUMMARIO IN INTERLINGUA

Un Rapide e Permanente Methodo de Tincturation Wright pro Chromosomas e Nucleos Cellular

Pro tincturar rapidemente chromosomas e nucleos de cellulas culturate super vitro e in suspensiones, preparatos fixate per medio de alcohol acetic e siccate in aere esseva neutralisate con ammoniaco liquide o dicarbonato de natrium, tractate con sero, e tincturate secundo Wright. Cellulas culturate ab tissu ovin, bovin, e murin esseva tincturate a bon successo. Iste preparatos es permanente. Illos pote esser examinate per microscopia conventional e photographate al discretion del investigator.

Scientific Notes

Coccidioidomycosis in 3 Coyotes

M. Straub, M.D.; R. J. Trautman, M.S.; J. W. Greene

SUMMARY

In 3 of 5 coyotes in Tucson, Ariz., small single primary foci of coccidioidomycosis were found in the lungs. In 2 animals, the tracheobronchial lymph nodes were affected and *Coccidioides immitis* could be cultured from them. The 3rd animal had morphologically and culturally negative lymph nodes. The primary focus contained typical organisms.

INTRODUCTION

Coccidioidomycosis has been observed in domestic animals, in animals in captivity, and in wild rodents.[1-5] However, we are not aware that it has been observed in coyotes. A few such observations will be presented here.

Materials and Methods

Five coyotes caught in traps in the vicinity of Tucson, Ariz., were examined for the presence of coccidioidomycosis. The animals were killed and necropsied immediately after death or after short periods of refrigeration. The lungs were examined radiologically and carefully dissected. The method has been described elsewhere.* During necropsy, with sterile precautions, tracheobronchial lymph nodes were cultured directly on Sabouraud's dextrose agar. Lesions in the lungs and lymph nodes and random pieces of liver and spleen were examined microscopically. The Gridley and the Grocott stains were used as fungus stains. A histologic diagnosis of coccidioidomycosis was made on the presence of spherules with endosporulation. Suspect cultures were tested by inoculation in mice.

Results

In 3 of the 5 animals examined, the morphologic diagnosis of coccidioidomycosis was made. Two had solitary pulmonary foci 2 mm. in diameter which radiologically were without calcification. The foci were situated subpleurally in the right apical lobe and in the right diaphragmatic lobe. They were well-circumscribed, small, pneumonic consolidations with an exudate containing histiocytes, polymorphonuclear leukocytes, lymphocytes, and plasma cells. In the lesions, the elastic tissue had largely disappeared; in one of them, the center was slightly necrotic; in the other, there was extensive caseation. Many typical spherules were found in or near the necrotic areas. In the im-

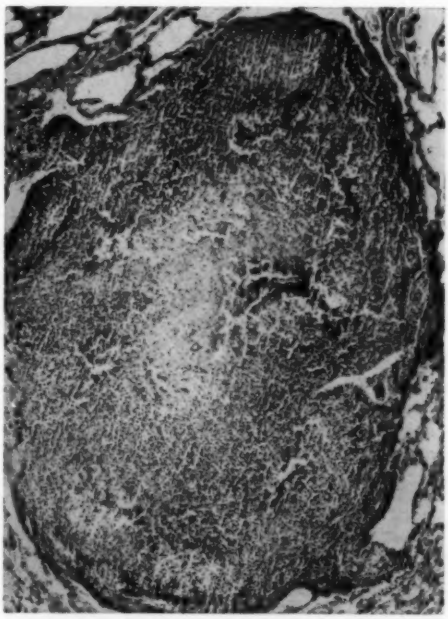

Fig 1—Granulomatous nodule, with central necrosis, in the lungs of a coyote. Spherules are numerous. Notice presence of helminthic larvae next to granulomatous lesion. H. & E. stain; x 65.

Received for publication Nov. 29, 1960.

From the Department of Animal Pathology, College of Agriculture of the University of Arizona, Tucson, where Mr. Trautman is research associate and Mr. Greene was, at the time of the study, a student. Dr. Straub is research associate, Clinical Laboratories, Jewish Hospital, Cincinnati, Ohio.

This project was supported in part by grants E-682 and E-576 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

* Straub, M., Trautman, R. J., Reed, R. E., and Schwarz, J., Location: Coccidioidomycosis in a Random Dog Population in Arizona.

mediate vicinity of one of the lesions, an unidentifiable helminthic larva was seen.

The 3rd positive animal had a solitary subpleural pneumonic lesion 0.4 cm. in diameter in the right apical lobe near the hilus. The exudate was similar to that in the other 2 positive cases, but neither

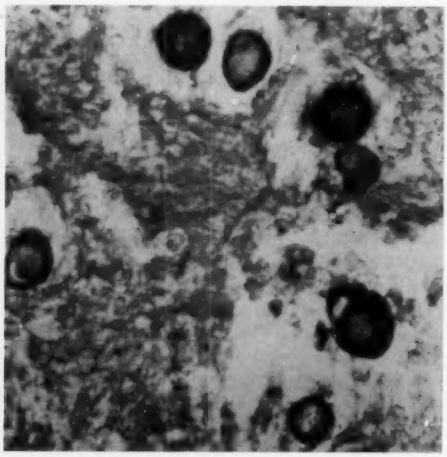

Fig. 2—Spherules in same nodule shown in Figure 1. Gridley stain; x 345.

necrosis nor spherules were found. In the tracheobronchial lymph nodes, however, there was fibroepithelioid granulomatous infiltration with patchy necrosis, with typical spherules in the necrotic areas, and in some epithelioid tubercles. Identical lymph node involvement was found in the carinal lymph node of one of the other positive animals. In the animals with morphologic lymph node lesions, Coccidioides immitis could be cultured from 2 to 3 tracheobronchial lymph nodes examined, but only in one case did inoculation in mice substantiate the diagnosis. The 3rd positive animal had morphologically and culturally negative tracheobronchial lymph nodes. The animals with the positive lymph nodes were females, one of which was pregnant. The positive animal with the negative tracheobronchial lymph node was male. Of the 2 negative animals, 1 was male, the other female.

Discussion

It is not astonishing that, in Arizona, a high percentage of desert animals like the coyote should be infected with C. immitis. In coccidioidomycosis, as in histoplasmosis, infection occurs aerogenously with spore-containing dust. The 1st lesions develop in the lungs and in the corresponding lymph nodes.[5] In one of the coyotes, the small single pulmonary focus and the affected tracheobronchial lymph node formed a "primary complex." In another positive animal, the primary complex remained "incomplete," since the lymph nodes were unaffected and no organisms could be isolated from them.

The 3rd positive coyote had extensive granulomatous lesions of the tracheobronchial lymph nodes in the presence of a solitary, well-circumscribed subpleural pneumonia focus without necrosis and without organisms. We have described similar lesions in canine histoplasmosis under the name of nodular pneumonia, and on the basis of positive lymph node cultures have come to the conclusion that they were histoplasmic primary foci in a prenecrotizing phase. We are inclined to regard the nodular pneumonic lesions in this 3rd coyote with its morphologically and culturally positive lymph nodes as a coccidioidal primary focus in a prenecrotizing phase. The lymph nodes were rather extensively affected. The animal was pregnant. In man, the tendency to dissemination of coccidioidal infection during pregnancy is well known.[6]

Heavy infection with spore-containing dust in histoplasmosis leads to multiple primary foci in the lungs.[4] In the epidemic type of the disease, the lungs may be peppered with primary foci. After incidental infection, a single pulmonary primary focus is the rule.[5] In the 3 described cases of coccidioidomycosis in coyotes, the pulmonary primary lesions were single. The constant exposure to which the coyote in the desert is prone is reflected in the high percentage of animals infected. The type of lesions, however, suggests incidental and not heavy infection.

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SUMMARIO IN INTERLINGUA

Coccidioidomycosis in 3 Coyotes

In 3 ex 5 coyotes in Tucson, Arizona, micre focos primari solitari de coccidioidomycosis esseva trovate in le pulmones. In 2 del animales, le nodos lymphatic tracheobronchial esseva affecite, e *Coccidioides immitis* poteva esser culturate ab illos. In le tertie animal, le nodos lymphatic esseva morphologicamente e culturalmente negative. Le focos primari contineva organismos typic.

Study of Chicken Sarcoma (Strain 13) by Vent Exposure

E. L. Stubbs, V.M.D.; F. G. Sperling, V.M.D.; A. M. Wallbank, M.S., Ph.D.

SUMMARY

Experiments were conducted by applying fresh chicken sarcoma (strain 13) tumor suspension by the vent-drop method and by the vent-brush method used for immunization against viruses. No tumors grew following application of tumor material to the vent. Later, all the vent-exposed chickens and an equal number of controls were inoculated intramuscularly to challenge any resistance produced by vent exposure. Both vent-exposed and control chickens developed tumors when inoculated with tumor cell suspensions intramuscularly. The vents of young chickens were not suitable for propagation of chicken sarcoma (strain 13), and vent exposure to this tumor did not raise the resistance of chickens to intramuscular injection of this tumor.

INTRODUCTION

A transmissible chicken sarcoma (strain 13), described as a virus-induced tumor by Stubbs and Furth [4] and reactivated from freeze-dried material by Stubbs and Sperling,[5] prompted experiments by vent exposure. It was considered desirable to determine whether this tumor would grow in the vents of chicks, and whether its application to the vent might confer any kind of resistance or immunity such as occurs with some viruses.

Infectious laryngotracheitis virus has been used for many years in a preventive vaccination procedure by brushing the virus on the mucous membrane of the vent as advocated by Beaudette and Hudson.[1]

Received for publication March 2, 1961.
From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.
Supported by funds from the Goldhaft Poultry Research Fund and Grant C-4610 from the United States Public Health Service.

The vent-drop method has been described by Hitchner and White[2] and Hitchner and Winterfield,[3] using a 19-gauge hypodermic needle on a syringe to place 1 drop of the vaccine on the unopened vent, or on the everted vent. A drop of the vaccine on the unopened vent seemed just as effective, because it was followed by a reflex movement of the vent that distributed the vaccine over the mucosa. The results indicated the drop method was superior to the brush method and had some distinct advantages. Later, Winterfield *et al.*[6] used the vent route and described a good immunity response in chickens vaccinated against Newcastle disease, infectious bronchitis, and infectious laryngotracheitis with live-virus vaccines. Chicken sarcoma (strain 13) is a virus-induced tumor, so the question arose as to whether this tumor could be transmitted by vent application and whether vent exposure might induce resistance.

Materials and Methods

Fresh chicken sarcoma was obtained from a chicken with a tumor in the muscles of the breast. Small pieces of tumor, in physiologic saline solution to keep the tumor material moist and cold, were placed in a Petri dish on ice and minced finely by cutting with small scissors. Approximately equal quantities of saline solution and tumor material were used. After mincing, a syringe with a 19-gauge hypodermic needle was used to remove small portions of tumor suspension. This procedure allowed small particles of tumor to be drawn into the syringe, along with fluid containing many cells.

The same syringe and needle were used immediately to discharge 1 drop of the tumor suspension on the vent of each of 17 chicks. This produced a reflex movement in the vent by which the drop was distribu ed over the vent mucosa as described by Hitchner and White.[2] The chicks were wing-banded for identification.

Immediately thereafter, 10 chicks from the same lot were wing-banded and inoculated intra-

muscularly on the left side of keel with 1 drop of the tumor suspension from the syringe and needle used in the vent exposure.

Results

All chicks were examined weekly. There was no evidence of tumor growth in the 17 chicks exposed by the vent drop methods, but in 2 of the 10 control chicks there was beginning tumor growth within 2 weeks. The tumors grew rapidly in the control chicks and were fatal within 2 months postinjection. After 4 weeks with no "takes" in the 17 vent-exposed chicks, resistance was challenged with fresh tumor suspension prepared from one of the control chicks and injected into the breast muscle. All of the 17 chicks developed tumors in the same pattern that had been found in the control chicks.

Experiments were then designed to try the older and more commonly used method of introducing the virus into the vent by the brush method used for many years in immunization against infectious laryngotracheitis.

Twenty White Leghorn chickens, 6 weeks old, were available for this experiment. The tumor suspension (strain 13) was obtained from a chicken that had had a tumor in the breast muscle for a month. Pieces of tumor were removed, minced by cutting with small scissors, placed in physiologic saline solution in a Petri dish, and kept on ice to keep cold and moist. The suspension was then passed through a fine tissue strainer. The strained tumor suspension was drawn into a syringe through a 19-gauge needle to remove any particles of tumor, permitting hypodermic injection of the tumor suspension. Half of this tumor suspension was used to brush into the vent mucosa of 10 chickens, and the other half was injected intramuscularly into the other 10 chickens to be used as controls.

A brush designed for infectious laryngotracheitis vaccination was dipped into the half of the tumor suspension prepared for this purpose, then brushed 4 times across the mucous membrane of the cloaca, which was everted by pressure between the thumb and forefinger. Finally, the vent was allowed to close over the brush to remove any remaining tumor suspension. The brush was dipped into the tumor suspension for exposure of each succeeding chicken. The tumor suspension in the syringe was used to intramuscularly inoculate the 10 remaining chickens to test the activity of the tumor suspension and the susceptibility of the chickens.

The chickens were examined each week by everting the vent to detect any tumor growth and by palpating the breast muscles to detect any swelling. No tumor growth was found in any of the vent-brush exposed chickens, but tumor growth was found in the breast muscles of controls. These results indicated that the vent is not a suitable place for propagation of this tumor.

Two weeks after the application of the tumor material to the vent, resistance was challenged by intramuscular injection. A new lot of fresh tumor material (strain 13) was prepared in the manner previously described for challenging by injection into the breast muscle of each chicken exposed previously by the vent-brush method. At the same time, 10 unexposed chickens from the same lot were inoculated in the breast muscles with the same tumor suspension as a test for activity and for comparison with the vent-exposed chickens.

Two weeks after challenge, 9 of the vent-brush exposed chickens had tumors growing at the point of injection in the breast muscles, and 9 of the chickens used as controls had similar tumors. Three weeks after challenge, all 10 of the vent-exposed chickens had tumors, as did 9 of the controls. No differences could be found in the size, consistency, or rapidity of growth of the tumors in vent-brush exposed chickens and the control chickens. These results indicated that the vent-brush exposure method, under the conditions of this experiment, did not produce any resistance or immunity.

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SUMMARIO IN INTERLINGUA

Studio de Sarcoma de Gallina (Racia 13) per Exposition Anal

Experimentos esseva conducite per applicar fresc suspensiones tumoric de sarcoma de gallina (racia 13) secundo le methodo de guttage anal e de brossage anal que es usate pro le immunisation contras virus. Nulle tumores cresceva post le application de material tumoric

al apertura anal. Plus tarde, omne le analmente exponite gallinas e un equal numero de gallinas de controlo esseva inoculate intramuscularmente pro provocar le resistentia que possibilemente habeva resultate del exposition anal. Tanto le gallinas experimental como etiam le gallinas de controlo disveloppava tumores quando illos esseva inoculate intramuscularmente con suspensiones de cellulas tumoric. Le anos de juvene gallinas non esseva usabile in le propagation de sarcoma de gallina (racia 13), e le exposition anal a iste tumor non augmentava le resistentia de gallinas contra le injection intramuscular de illo.

Meetings • • •

• • • International Conference of Protozoologists. Prague, Aug., 1961. John O. Corliss, Secretary, Society of Protozoologists, University of Illinois, Urbana.

• • • Seventh Inter-American Congress of Radiology. Sao Paulo, Sept. 3-10, 1961. Dr. Walter Bomfim-Pontes, Secretary General, Rua Cesario Motta 112, Sao Paulo, Brazil.

• • • Symposium on Elimination of Infectious Diseases Affecting Laboratory Animal Colonies. Prague, Sept. 4-8, 1961. International Committee on Laboratory Animals, Dr. W. Lane-Petter, Secretary General, c/o M.R.C. Laboratories, Woodmansterne Rd., Carshalton, Surrey, England.

• • • International Tuberculosis Conference. Toronto, Sept. 10-14, 1961. Dr. C. W. L. Jeanes, Secretary General, 265 Elgin Street, Ottawa, Canada.

• • • European Association for Animal Production, 2nd Symposium on Energy Metabolism of Farm Animals. Wageningen, Netherlands, Sept. 11-15, 1961. Corso Trieste 67, Rome, Italy.

• • • Second International Symposium on Chemotherapy. Naples, Sept. 14-17, 1961. Dr. P. Rentchnick, Secretary General, Case 229, Geneva 4, Switzerland.

• • • Fifth International Embryological Conference. London, Sept. 18-21, 1961. Dr. L. Brent, University College, Department of Zoology, Gower St., London, W.C. 1., England.

• • • International Association of Veterinary Anatomists. Vienna, early September, 1961. Dr. Robert Getty, Vice-President of the Association, Prof. of Veterinary Anatomy, Iowa State University, Ames.

• • • International Committee on Laboratory Animals. Prague, Sept., 1961. Dr. W. Lane-Petter, Secretary General, c/o M.R.C. Laboratories, Woodmansterne Rd., Carshalton, Surrey, England.

• • • International Dairy Federation. Luxembourg, Sept., 1961. 10 rue Ortelius, Brussels 4, Belgium.

• • • Council for International Organizations of Medical Sciences. Paris, Oct. 6-10, 1961. 6 rue Franklin, Paris 16e, France.

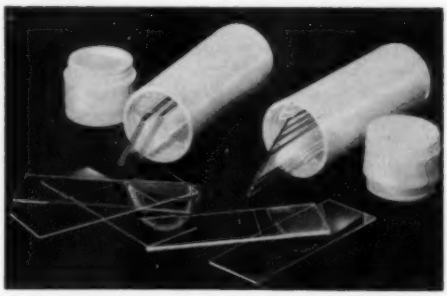
• • • International Association of Milk and Food Sanitarians. Atlanta, Ga., Oct. 20-22, 1961. H. L. Thomasson, Box 437, Shelbyville, Ind.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave, Chicago 5, Illinois.

Plastic Mailer for Microscope Slides

A new, inexpensive, reuseable polypropylene container has been developed by Double D Plastics Co. for protecting microscope slides against contamination and breakage during mailing or storage. The new container has been approved by the United States postal authorities for mailing.



The tube, liquid—and air-tight—can be sterilized in boiling water or autoclaved without undue distortion. It has 2 sets of internal slots for either 4 single slides or 2 pairs of slides inserted together. A tight-fitting screw cap seals the container.

Neoprene Flooring

A special neoprene-cement compound which helps prevent bacterial and virus contamination is now being used as a laboratory flooring. This syn-

thetic rubber flooring is a quarter-inch thick and is free from joints, seams, or cracks, thus eliminating viruses and bacteria in small cracks and crevices in the floor. The rubber surface is not affected by animal excrements, steam cleaning, or germicidal agents. This flooring is a product of Crossfield Products Corporation.

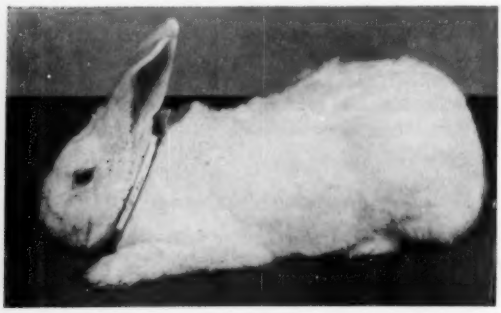
Animal Identification Method

Positive, at-a-glance identification of animals in laboratories, hospitals, and kennels can now be achieved with the new Animal Care Ident-A-Band by Hollister Inc. Important information about the animal is recorded on an insert card and sealed inside the band. Information can't accidentally fall out or be scratched out, and the band is water-resistant.

Ident-A-Band consists of a slim strip of soft vinyl concealing a tough core of Mylar (tensile strength equal to steel) which resists breaking or stretching by the most active animal.

A reel dispenser holds 200 feet of Ident-A-Band. Every reel includes more than 200 locket-type clip fasteners. Once the clip is closed it seals permanently and cannot be reused. This one-use disposable feature eliminates possibility of mixup in handling.

When boarding pets in a kennel, the band can be attached in the owner's presence and removed when the animal is released. This will give the owner added assurance that he is receiving the right pet, especially in the case of young animals. These bands can also be adapted for special laboratory uses. Colored insert cards allow easy project classification; duplicate band attached to cage provides special correlation; and because band is lightweight and non-allergenic, animal is comfortable even when medications or dressings are used.



Disposable Surgical Masks

Two lightweight, disposable, surgical masks, capable of more than 90% bacterial filtration, are now available nationally through surgical supply houses. Both masks were developed by Minnesota Mining and Manufacturing Co.

One mask is made of nonwoven fabric with filtration through its entire surface; the other is flexible plastic with a nonwoven filtering area. The masks have stabilized porosity, assuring uniform filtering. The former fit of the masks prevents the escape of air around the edge, eliminating eyeglass fogging and giving an unobstructed field of vision. Since neither mask touches the nostrils or mouth, the masks remain drier and permits normal conversation.

Animal Bedding Material

An animal bedding material that neutralizes odors and inhibits the growth of bacterial organisms has been developed by the C. P. Hall Co.

The base material for the bedding is finely ground, clean, dustless corn cobs of granule size. This is treated with a patented formulation which retards the hydrolytic and bacterial decomposition of liquid and solid waste matter and eliminates the ammonia odors normally present. The lightweight material is free-flowing and does not mat or cake.

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